Biochemical and Molecular Characterization of some agriculturally important microbes

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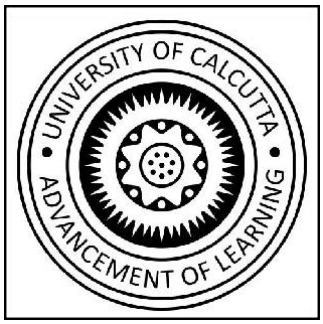

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DECLARATION BY THE STUDENT

I hereby declare that the work which is being presented in this dissertation entitled " **Biochemical and Molecular Characterization of some agriculturally important microbes**" in partial fulfilment of the requirements for the award of the degree of Masters of Science in Biotechnology is an authentic record of my own work carried out during the period from 1st January2025 to 31st May under the supervision of Prof. (Dr.) Krishnendu Acharya Department of Botany, University of Calcutta.

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LIST OF FIGURES

Figure No.	TITLE	PAGE NO.
1.	Zinc Solubilizing activity of ten bacterial isolates	25
2.	Phosphate Solubilizing activity of ten bacterial isolates	26
3.	Potassium Solubilizing activity of ten bacterial isolates	27
4.	Nitrogen Fixing ability of ten bacterial isolates	28
5.	Indole Acetic Acid production of bacterial isolates	29
6.	Ammonia Production results of bacterial isolates	30
7.	Gram Characterization of sample isolates	33
8.	Capsule staining of sample isolates	34
9.	Endospore staining of sample isolates	35
10.	Culture slants of sample isolates	36
11.	Motility test of ten bacterial isolates	36
12.	IMViC test results of sample isolates	38
13.	Amylase Test results of sample isolates	39
14.	Cellulose utilization of sample isolates	40
15.	Carbohydrate Utilization of sample isolates	41
16.	Results of urease test	43
17.	Results of TSI test	44
18.	16s rRNA bands of isolated samples after PCR amplification	44

LIST OF TABLES

Table No.	TITLE	PAGE NO.
1.	Plant Growth promoting activities of sample isolates.	31
2.	Culture Characteristics and Colony morphology of sample isolates	31
3.	Microscopic characteristics of sample isolates	32
4.	Biochemical and carbon source utilization characters of sample isolates	42

ABBREVIATIONS

SL No.	ABV	Meaning
1.	IAA	Indole Acetic Acid
2.	FYM	Farm Yard Manure
3.	PGPB	Plant Growth Promoting Bacteria
4.	BNF	Biological Nitrogen Fixation
5.	PSM	Phosphate Solubilizing Microorganism
6.	KSM	Potassium Solubilizing Microorganism
7.	ZnSM	Zinc Solubilizing Microorganism
8.	PSB	Phosphate Solubilizers
9.	KSB	Potassium Solubilizers
10	ZnSB	Zinc Solubilizers
11.	N-Fix	Nitrogen Fixer
12.	PGPR	Plant Growth Promoting Rhizobacteria
13.	NA	Nutrient Agar
14.	NB	Nutrient Broth
15.	TSI	Triple Sugar Iron Agar
16.	MSM	Mineral Salt Media
17.	PCR	Polymerase Chain Reaction
19.	dNTP	Deoxynucleotide Triphosphate
20.	DNA	Deoxyribonucleic acid
21.	Fig	Figure

ABSTRACT

Due to the adverse alterations in soil conditions resulting from imprudent and hazardous applications of chemical fertilizers, there exists a pressing necessity for environmentally sustainable soil management through the identification and isolation of potential strains that exhibit plant growth-promoting activities, thereby facilitating the enhancement of macronutrient uptake, including nitrogen (N), zinc (Zn), potassium (K), and phosphate (P), to safeguard the integrity and health of the soil ecosystem. The objectives of this study encompass the isolation of microorganisms suitable for use as bioinoculants, alongside an examination of the characteristics of microbes capable of augmenting the uptake of macronutrients such as nitrogen (N), phosphate (P), potassium (K), and zinc (Zn).

In pursuit of proficient plant growth-promoting strains exhibiting a diverse array of activities, a total of ten bacterial strains were isolated from soil samples. Each isolate was rigorously screened based on morphological and biochemical characteristics. Subsequently, the isolates were assessed for their plant growth-promoting properties, which included the production of ammonia (NH3), indole acetic acid (IAA), phosphate solubilization, zinc solubilization, nitrogen-fixing capacity, and potassium solubilization. Out of ten isolated samples all of them showed Nitrogen Fixing capability, seven of the samples showed phosphate solubilizing capability, two of them showed potassium solubilizing capability and only one showed Zinc solubilizing activity. The sample isolates were then characterized through various morphological and Biochemical tests.

Keywords: Bio-inoculants, Biofertilizers, Zinc Solubilizers, Potassium Solubilizers, Phosphate Solubilizers, Nitrogen Fixers

CONTENTS

SL NO.	TITLE	PAGE NO.
1.	Acknowledgement	I
2.	List of Figures	II
3.	List of Tables	III
4.	Abbreviations	IV
5.	Abstract & Keywords	V
6.	Introduction	1-8
7.	Review of Literature	9-17
8.	Materials And Methodology	18-24
9.	Results	25-44
10.	Discussion	45
11.	Conclusion	46
12.	Appendix	47-49
13.	References	50-54
14.	Plagiarism report	55

INTRODUCTION

The industrial green revolution took place during the mid-20th century that signified a monumental transformation in agricultural methodologies, introducing the use of chemical fertilizers that were intended to significantly enhance soil fertility. The widespread application of pesticides was designed to effectively control pests and diseases, along with the mechanistic upliftment of agricultural practices that was aimed to increase overall productivity of crops (Van Zanden, 1991). This movement was primarily aimed to address the present global food shortages that was complemented with agricultural productivity in a large scale. But this revolutionary approach has faced substantial criticism for its adverse environmental and health effect, which include notable soil degradation, significant water pollution, and loss of biodiversity across various ecosystems. Therefore, the world has been changed towards a trend of organic farming practices, which gives strong emphasis on the utilization of natural and sustainable practices that can ameliorate soil health, enhance biodiversity, and provide essential ecosystem services that can support both agriculture and the environment. Some factors may be responsible for shifting conventional inorganic farming to more sustainable organic farming (Reganold, 1998; Mondelaers, 2009; Costa, 2014).

- Environmental concerns that encompass critical issues such as soil degradation, the alarming levels of water pollution, and the significant loss of biodiversity that threatens ecological stability and sustainability;
- Health benefits that are inclusive of a reduced exposure to harmful chemical pesticides that pose risks to human health and safety;
- An increased consumer demand for organic products, which is driven by a heightened awareness among consumers and their willingness to pay premium prices for food that is perceived as healthier and more environmentally friendly;
- Moreover, supportive government policies and regulations that facilitate organic farming, including comprehensive certification programs and financial subsidies aimed to encourage farmers in organic practices.

There is a negative impact of conventional inorganic agricultural methods on the preservation of biodiversity, the general health of the environment, and the welfare of

farming communities, which promoted organic agriculture in a comprehensive approach (Hole, 2005) that includes the use of natural products of ecosystem like cow dung, vegetable litters etc. that gradually improve soil fertility over time. Organic farming incorporates methods like crop rotation and biological pest control with the thoughtful application of natural fertilizers like compost and manure that can maintain sustainably and increase crop yields (Gamage, 2023).

Organic farming has multifaceted aims (Meena, 2020; Ramesh, 2005):

- To improve soil health with sustainable long-term soil fertility.
- To increase biodiversity and strengthen ecosystem services that are vital for sustainability of agriculture.
- To reduce environmental pollution and mitigate health risks that may arise from conventional farming practices.
- To enhance the nutritional quality of crops, thereby benefiting consumers and public health.
- To support local cropping systems and stimulate rural development initiatives.
- To utilize plant and animal waste by recycling them for replenishing of essential soil nutrients, thereby promoting sustainable agricultural practices.

Different international organizations, such as the Food and Agriculture Organization (FAO) and different international agencies like the United States Department of Agriculture (USDA), promoted and facilitated organic farming though it is subjected to scrutiny and examination (Luttikholt, 2007). These institutions have established organic standards, providing essential technical assistance to farmers, and facilitating the integration of organic products into global markets. Additionally, they provide support in the development of sustainable agricultural practices, including organic farming, as a viable means to alleviate hunger, enhance food security, and protect the environment from degradation (Joachim, 2006).

Despite the numerous benefits associated with organic farming, it is important to acknowledge that this approach faces several formidable challenges, which include higher production costs, lower crop yields compared to conventional farming practices, and limited access to markets for organic products. However, it is noteworthy that a growing number of farmers and consumers are increasingly embracing organic practices, driven by substantial concerns regarding environmental sustainability, public health implications, and the overarching issue of food security in our societies.

COMPONENTS OF ORGANIC FARMING:

Organic Fertilizers:

1. Bulky Organic Manures

Applied in large quantities consisting of Farmyar Manure (FYM), Green manure, Compost etc.

1.1. Farmyard Manure:

Farmyard manure is a naturally occurring organic fertilizer that is created from animal waste, including cow dung, urine, straw, hay, and other farm residues. The ability of this bulky organic manure to improve soil fertility and structure makes it one of the most popular components in agriculture. FYM improve soil health by modifying aeration, texture, and water retention capacity (Pinto, 2017). Compared to synthetic fertilizers, the manure has comparatively low in concentrations of several vital nutrients, such as potassium, phosphorus, and nitrogen. A well decomposed FYM contains 0.5% N_2, 0.2% P_2O_5 and 0.5% K_2O. Urine contains 1% N_2 and 1.35% K_2O. *Actinomycetes* and bacteria are actively involved in the decomposition process (Lacey, 1997). Urine primarily contains nitrogen in the form of urea, which is lost through volatilization (Dewes, 1998).

1.2. **Compost:** An integral part of organic farming is compost that serves as a necessary organic input to enhance the fertility and health of the soil. It is formed by microbial breakdown of organic matters like plant waste, animal dung, crop residues, and other biodegradable materials (Chen, 2011). Composting transforms these organic materials into a rich, humus-like, nutrient-dense material that can be added to soil to improve its quality that contributes to the development of healthy soils and promotes sustainable crop production. As microbes decompose organic material they convert complex substances like proteins, carbs, and fats into simpler forms that plants can use as they cannot break down organic matter. These nutrients including nitrogen (N), ammonium (NH_4^+) or nitrate (NO_3^-), are forms of nitrogen that are released by bacteria and are necessary for plant growth. Organic phosphorus (P) is also available to plants by the action of bacteria and fungi (Chen, 2011).

Microbes also aid in the breakdown and accessibility of other minerals, such as potassium (K) and calcium (Ca), for plants (Chandana, 2013). There are two following methods of composting:

- **Aerobic Composting :** Among composting techniques, aerobic composting is the most common. It involves microorganisms that flourish in oxygen-rich environments breaking down organic materials. Turning the compost pile on a regular basis ensures proper aeration and oxygen availability for the microorganisms involved in decomposition. Mesophilic organisms (20–45°C) first proliferate quickly on easily accessible sugars and amino acids, then produce heat through their metabolism, which in turn inhibits their activity. The material's temperature is then raised to 62°C or higher by a few thermophilic organisms (between 50°C and 70°C or higher) continuing the process (Mehta, 2018). Since pathogens and weed seeds are killed by the heat, this optimal heating phase is crucial for the compost's quality. After this stage, there is a curing stage during which more bacteria and fungi starts to proliferate. The primary material broken down by these organisms is cellulose and hemicellulose found in plant cell walls. A buffer against the harmful effects of organic acids on plants is provided by the compost's curing process. At the end, the compost becomes less biologically active and more uniform as the temperature drops, even though mesophilic organisms recolonize it (Chen, 2011).

- **Anaerobic Composting :** The process of composting without oxygen is known as anaerobic composting. Microbes that do not require oxygen for decomposition are used in anaerobic composting, in contrast to aerobic composting, where microorganisms flourish in environments rich in oxygen (Mehta, 2018). A distinct group of microorganisms, including anaerobic bacteria and methanogens, perform anaerobic composting as opposed to aerobic composting. These microbes produce methane (CH_4), carbon dioxide (CO_2), and a tiny amount of heat as byproducts of breaking down organic

materials in a closed, oxygen-deprived environment (Chen, 2011). It may take longer time for the organic material to completely decompose and the process is generally slower than aerobic composting.

2. **Bio-gas Slurry** : Bio-gas slurry, also known as organic fertilizer, is a sustainable and eco-friendly byproduct of the complex process of anaerobic digestion, in which a variety of organic materials, including animal manure, agricultural crop residues, kitchen waste, and other biodegradable materials, break down in an oxygen-free environment to produce biogas, a gaseous mixture mainly consisting of methane and carbon dioxide, and the nutrient-rich slurry (Kumar, 2015). This high nutrient rich slurry contains vital components like potassium (K), phosphorus (P), and nitrogen (N), that makes it an incredibly valuable and efficient fertilizer source which is especially beneficial for agricultural applications. The slurry contains a significant amount of partially decomposed organic matter, which helps to improve soil structure, water-holding capacity, and aeration—all of which together promote soil health and biodiversity. This slurry is also used to reduce soil erosion and increase crop yields, making it a sustainable and practical substitute for traditional chemical fertilizers, which often contribute to environmental problems such as water pollution, soil degradation etc (Kumar, 2022).

3. **Green Manure:** In order to increase soil fertility and structure, certain plants—typically legumes—are cultivated and then added to the soil while it is still green or shortly before it gives flowers. This technique is known as "green manuring". These plants, also referred to as green manure crops, are usually grown to improve the soil's fertility, structure, texture, and other physical characteristics to boost biological activity and enrich the soil with organic matter and to improve the soil's nutrient content and overall health (Mamiev, 2019). Additionally, it prevents soil erosion and offers soil cover for no-Tillage. These kind of manure crops are typically non-woody annual plants that grow quickly (Cherr, 2006). Good green manure crops don't need any special soil preparation or fertilizers of any kind, and they should have a long vegetative growth phase and be able to withstand a wide range of climatic and environmental conditions. A variety of plant species could

be used as green manure crops. These crops could be divided into two groups (leguminous crops and non-leguminous crops) (Ashraf, 2004).

4. **Vermicompost :** Vermicomposting constitutes an exceptionally effective and environmentally sustainable technology for the management of organic waste, as well as for enhancing agricultural productivity in various contexts. By facilitating the conversion of organic waste materials into a nutrient-dense soil amendment, it plays a pivotal role in promoting soil health and advancing environmental conservation efforts on a broader scale (Lim, 2015). The process of vermicomposting can be characterized as a bio-oxidative phenomenon that inherently involves two principal decomposers, namely earthworms and a diverse array of microorganisms. This intricate process encompasses the ingestion, subsequent digestion, and ultimate excretion of organic matter by earthworms, culminating in the production of vermicast, which is a finely divided substance resembling humus that is abundant in essential plant nutrients (Rostami, 2011). Microorganisms, in this context, are critical participants as they engage in the breakdown of complex organic compounds, thereby facilitating the transformation of nutrients into forms that are more readily available for plant uptake. Numerous species of earthworms have demonstrated considerable efficacy in the practice of vermicomposting, with the most frequently utilized species being *Eisenia fetida*, commonly known as the red wiggler, and *Eudrilus eugeniae*, referred to as the African nightcrawler (Reinecke, 1992). These particular species have gained preference among practitioners due to their remarkably high reproductive rates, their capacity to consume a diverse range of organic materials, and their exceptional efficiency in generating high-quality compost. The application of vermicompost has been shown to significantly enhance soil structure, improve aeration, and promote moisture retention, while simultaneously increasing microbial activity that is indispensable for supporting optimal plant growth (Moradi, 2014). This organic amendment is replicated with beneficial microbial flora that plays a fundamental role in processes such as nitrogen fixation and the solubilization of various nutrients necessary for plant development (Gopal, 2012). Furthermore, vermicompost contains a multitude of essential plant growth-promoting hormones, including auxin, cytokinin, gibberellins, ethylene, and

abscisic acid, all of which contribute to the physiological processes that govern plant growth and development (Arancon, 2005). The microbial population contained within vermicompost is known to exhibit antagonistic effects against pathogenic microorganisms through the production of an array of antipathogenic compounds, including but not limited to siderophores, antibiotics, chitinase, and cyanide, thereby providing a protective barrier for plants against a myriad of pests and diseases that may threaten their health and productivity (Mu, 2017; Yatoo, 2021).

5. **Bio-fertilizers:** Biofertilizers are natural substances that include living microorganisms which can improve the nutrient availability and supply to plants. Bacteria, fungi, and algae are some of the microorganisms that interact with the soil and plant roots to enhance nutrient uptake and encourage robust plant growth (Kumar, 2017). Biofertilizers, as opposed to chemical fertilizers, complement the soil microbiome and promote sustainable farming methods by interacting with nature (Mahanty, 2017). Furthermore, biofertilizers improve microbial diversity, which lowers the need for external nutrient supplementation and improves soil health. Biofertilizers increase plant growth by promoting the synthesis of growth-promoting compounds, fixing nitrogen naturally, and solubilizing phosphorus (Garcia-Fraile, 2015). The microorganisms found in biofertilizer help to build soil organic matter and restore the soil's natural nutrient cycle. It outperforms farmyard manure and other types of manure in terms of N P: K. Biofertilizers contains about 3.5-4% nitrogen, 2-2.5% phosphorus and 1.5% potassium.

6. **Bioinoculants in Organic Farming** : At a global level, the repercussions of agricultural practices such as chemical fertilization can inflict significant damage upon the environment. Inoculation represents one of the most critical and sustainable methodologies in agriculture, as microorganisms form symbiotic relationships with plants and facilitate plant development through various advantageous traits (Souza, 2015). Interactions between plants and microbes in the rhizosphere serve as pivotal determinants of plant vitality, productivity, and soil fertility. Plant growth-promoting bacteria (PGPB) are specific bacterial strains capable of enhancing plant growth while simultaneously safeguarding plants against diseases and abiotic stresses through a diverse array of mechanisms. The

rhizosphere constitutes the zone encompassing the soil associated with plant roots and frequently extends a few millimeters beyond the root surface, representing a crucial environment for the interactions between plants and microorganisms (Lynch, 1990; Gray and Smith, 2005). The mechanisms by which bacteria can affect plant growth exhibit variability among species and strains; thus, it is generally acknowledged that there is no singular mechanism universally applicable to the promotion of plant growth. Nevertheless, the success and efficacy of PGPB as inoculants for agricultural crops are contingent upon a multitude of factors, including the capacity of these bacteria to colonize plant roots, the exudation from plant roots, and the overall health of the soil (Alquéres et al., 2013; Beauregard et al., 2013). The implementation of effective inoculants can be regarded as a vital strategy for sustainable management and for mitigating environmental challenges by reducing the reliance on chemical fertilizers (Alves et al., 2004; Adesemoye et al., 2009; Hungria et al., 2010, 2013).

7. Nitrogen Fixing Biofertilizers:

One of the most important macronutrients for plant growth and development is nitrogen (N) which is found in nitrogen-fixing biofertilizer formulations that can transform atmospheric nitrogen into forms that plants can absorb, like ammonia (NH_3). This process, called biological nitrogen fixation (BNF), is made possible by the nitrogenase enzymes found in particular microbes (Bhat, 2015). Biofertilizers enhance the nutrient profile of the soil by adding nitrogen that has been biologically fixed to it. As a result, fewer synthetic nitrogen fertilizers are required, which may cause nutrient imbalances and soil acidification. These biofertilizers fall into three categories based on their mode of interaction with plants:

7.1 Symbiotic Nitrogen Fixation: *Rhizobium* and other symbiotic nitrogen-fixing microorganisms establish mutualistic relationship with legumes, beans, and peas. These bacteria can grow within root nodules, which are specialized structures where nitrogen fixation takes place, after colonizing plant roots. The host plant reciprocates by providing the bacteria with carbohydrates and a favourable environment for survival.

Nitrogen Fixation by *Rhizobium*: The exchange of chemical signals initiates the process. *Rhizobium* is attracted to flavonoids, which are secondary metabolites secreted into the rhizosphere by legume roots. The plant's root hair cells recognize the Nod factors (lipochitooligosaccharides) secreted by *Rhizobium* through particular receptors (Goethals et al., 1992) (Spaink et al., 1991). Nod factors cause root hair to curl. In order for *Rhizobium* to colonize the root cortex, the plant cell membrane invaginates to form a tubular structure known as the infection thread through which *Rhizobium* can enter (Price et al., 1992). When *Rhizobium* penetrates the cortex and induces localised cell division in the root cortex and pericycle, root nodules are produced. Within these nodules, *Rhizobium* develops into nitrogen-fixing bacteroids encased in membrane structures known as symbiosomes. Inside the bacteroids found in the root nodules, nitrogen fixation takes place. The nitrogenase enzyme complex catalyses the conversion of atmospheric nitrogen (N_2) into ammonia (NH_3). (Mylona, 1995).

There are few genes (*nod*, *nif*, *fix*) that are responsible for the nitrogen-fixation process and also acts as a common genetic determinant for *Rhizobium* (Fischer, 1994).

7.2 Non-Symbiotic Nitrogen Fixation : Non-symbiotic nitrogen fixation is a process whereby free-living microorganisms in the soil convert atmospheric nitrogen (N_2) into ammonia (NH_3), a form accessible to plants. In contrast to symbiotic nitrogen fixation, free-living nitrogen fixers can carry out this crucial ecological task without the assistance of a host plant. They are essential for preserving ecosystems' nitrogen balance, especially in soils with low nitrogen concentrations. These organisms are mainly used in fertilizers for non-leguminous plants (Delwiche, 1956).

Biochemistry if N_2 fixation: The nitrogenase enzyme complex, which is in charge of nitrogen fixation in free-living microorganisms, catalyses the reduction of atmospheric nitrogen (N_2) into ammonia (NH_3) (Dent, 2018). The energy-intensive reduction of nitrogen depends on the passage of electrons from dinitrogenase reductase to dinitrogenase, which is made possible by ATP (Idris, 2024). When atmospheric nitrogen (N_2) attaches to the active site of nitrogenase enzyme it is reduced producing ammonia (NH_3). After being released into the environment, ammonia can either be absorbed by plants or transformed by microbes into other nitrogenous compounds. ree-living microorganisms that fix nitrogen include cyanobacteria (*Anabaena, Nostoc*) and bacteria (*Azotobacter, Clostridium*).

7.3 Associative Symbiotic Nitrogen Fixation : Associative nitrogen fixation is a process in which nitrogen-fixing bacteria, usually in the rhizosphere (the area of soil directly impacted by root secretions), form a loose, non-symbiotic relationship with plants and convert nitrogen into ammonia (Elmerich, 1992). Associative nitrogen-fixing bacteria can colonize the root cortex's intercellular spaces, root hairs, or the plant's root surface. Root exudates, which include organic substances like sugars, amino acids, and flavonoids, draw microorganisms to the rhizosphere. *Azospirillum*, which is mostly found with non-leguminous plants, fixes nitrogen and produces growth regulators to help plants grow (James, 2000). These bacteria improve plant growth by transforming atmospheric nitrogen (N_2) into ammonia (NH_3), which plants can absorb and use, even though they do not form specialized

structures like nodules. In order to manage soil fertility and practice sustainable agriculture, this kind of nitrogen fixation is essential.

8. Phosphate solubilizing Biofertilizers:

Phosphorus (P), is widely recognized as the second most critical macronutrient that plants necessitate for their optimal growth and development, following behind nitrogen in terms of its importance. It plays an indispensable role in virtually every facet of plant growth and development, influencing processes at the molecular level as well as a myriad of physiological and biochemical activities that are vital for plant health, such as photosynthesis, the development and expansion of roots, the fortification of stalks and stems, the formation of flowers and seeds, the maturation of crops, and the overall quality of the harvested produce, in addition to facilitating energy production, storage, and transfer reactions, promoting root growth, enabling cell division and enlargement, and assisting in the transportation of genetic traits from one generation of plants to the next (Sharma, 2013; Kumar, 2018; Khan 2009; Satyaprakash, 2017; Walpola, 2012). Nevertheless, the accessibility of soluble forms of phosphorus in the soil for uptake by plants is severely restricted due to its tendency to become fixed in the form of insoluble phosphates associated with iron, aluminium, and calcium compounds present in the soil matrix. In a general assessment, the phosphorus content found within the soil typically hovers around 15% by weight, yet the fraction of phosphorus that is actually available for plant uptake stands at a mere 0.1%, which indicates a significant discrepancy. Notably, soil across India exhibit a widespread deficiency in phosphorus content, which poses a considerable challenge for agricultural productivity. Substantial quantities of phosphate fertilizers are routinely applied during agricultural practices to mitigate this issue; however, a substantial portion of this applied phosphorus becomes immobilized due to its heightened reactivity with Al^{3+} and Fe^{3+} ions in acidic soils, as well as with Ca^{2+} in both calcareous and neutral soils, leading to precipitation and rendering the phosphorus unavailable for plant absorption (Thomas, 2005).India is currently the world's third-largest fertilizer consumer, demonstrating the extent of fertilizer use in the country. As the majority of the inorganic phosphorus fertilizers that are applied tend to bind with soil elements, this interaction results in a decrease in soil fertility, the disruption of microbial communities, and ultimately a marked reduction in overall crop productivity (Eichler-Löbermann, 2007). Furthermore, this situation gives rise to

significant environmental concerns, such as the contamination of groundwater resources and the phenomenon of water eutrophication, both of which have detrimental effects on ecosystems.

An additional significant source of phosphorus in soil ecosystems is derived from organic matter, which contributes to the overall nutrient dynamics within the soil environment. The majority of organic phosphorus present in soils is synthesized by microbial activity, and it is subsequently absorbed by plants in the form of soil phytate, which is chemically identified as inositol phosphate. It is noteworthy that soil phytate constitutes approximately 50% of the total organic phosphorus content, and it possesses a high degree of stability within the soil matrix. Phospholipids, phosphomonoesters, phosphodiesters, phosphotriesters, and different nucleic acids are examples of other organic phosphorus forms. Phosphate solubilizing microorganisms (PSMs), which are integral components of the natural phosphorus cycle, play a crucial role in the solubilization of insoluble forms of inorganic phosphorus as well as the mineralization of its organic forms, thereby rendering phosphorus more readily accessible to plants for uptake and utilization (Tian, 2021).

Phosphorus solubilizing microorganisms (PSMs) employ a variety of strategies to enhance the availability of phosphorus for uptake by plants, which may include techniques such as lowering soil pH, chelation, and mineralization processes (Tian, 2021). Among these various strategies, the organic acid theory, which posits that lowering soil pH can enhance phosphorus availability, stands out as the most widely recognized and accepted approach within the scientific community. The fundamental mechanism underlying the solubilization of phosphorus in the soil is the reduction of soil pH, which is facilitated by the microbial production of organic acids or through the release of protons into the soil environment. In alkaline soils, phosphate ions have a propensity to precipitate and form calcium phosphates, which include rock phosphate varieties such as fluorapatite and francolite, both of which are characterized by their insolubility in soil. However, the solubility of these phosphates tends to increase as soil pH decreases. PSMs enhance the availability of phosphorus by synthesizing organic acids that effectively lower the soil pH, thus promoting phosphorus solubilization. These organic acids are primarily products of microbial metabolic processes, predominantly generated through oxidative respiration or

fermentation pathways, particularly when glucose serves as the carbon source. The specific type and quantity of organic acids produced can vary significantly among different microbial organisms. The efficiency of phosphorus solubilization is contingent upon both the strength and the chemical nature of the acids produced. Additionally, tri-carboxylic and dicarboxylic acids have been shown to exhibit greater effectiveness in solubilizing phosphorus when compared to monobasic and aromatic acids, while aliphatic acids have also demonstrated superior efficacy in phosphate solubilization relative to phenolic, citric, and fumaric acids. The organic acids that are particularly effective in solubilizing phosphates include, but are not limited to, citric, lactic, gluconic, 2-ketogluconic, oxalic, glyconic, acetic, malic, fumaric, succinic, tartaric, malonic, glutaric, propionic, butyric, glyoxalic, and adipic acid (Schneider et al., 2019; Jayakumar et al., 2019; Kalayu, 2019; Azaroual et al., 2020). Research indicates that Gram-negative bacteria possess a greater capacity for dissolving mineral phosphates compared to their Gram-positive counterparts, largely due to their ability to release a diverse array of organic acids into the surrounding soil environment.

Phosphate mobilizing microorganism like Mycorrhizal fungi have been shown to significantly enhance the phosphorus supply to higher plants in a manner that surpasses the capabilities of other microbial entities. Mycorrhizae establish mutualistic symbiotic relationships that are predicated on bidirectional nutrient transfer between soil fungi and the root systems of vascular plants, which is essential for optimizing nutrient exchange (Chiu and Paszkowski, 2019; Genre et al., 2020). In this symbiotic arrangement, the plant provides the fungi with carbohydrates in the form of sugars that are produced via photosynthesis, while the extensive hyphal network formed by the fungi enhances the plant's ability to absorb both water and essential nutrients from the surrounding soil. This heightened efficiency in nutrient uptake is primarily attributable to the substantial surface area of the arbuscular mycorrhizal (AM) fungi hyphal network, which significantly increases the absorptive capacity of the plant's root system (Shi et al., 2021). Additionally, the fungal partners generate extraradical mycelium, which can extend extensively throughout the soil environment, thereby substantially enlarging the area available for root absorption. The interaction also increase the root to shoot area ratio by increasing number of roots and length of roots (Hammelehle et al., 2018; Lynch, 2019; Zhang Z. et al., 2021). Notably, phosphorus is classified as a highly immobile element because of its

propensity to be readily absorbed by soil particles, which causes a phosphate-free zone to grow rapidly surrounding plant roots. Beyond this depletion zone, the extraradical hyphae efficiently absorb bioavailable phosphate that the plant would not otherwise be able to obtain. There is compelling evidence to suggest that AM fungi interact synergistically with rhizosphere microorganisms, thereby enhancing the establishment of bacterial populations, many of which secrete phosphate-solubilizing enzymes that facilitate nutrient availability. These intricate interactions result in positive synergistic effects, ultimately leading to enhanced plant nutrition, growth, and overall survival in various environmental conditions.

9. Potassium Solubilizing biofertilizers:

Potassium (K), stands as one of the most crucial mineral elements that can be located within the various layers of soil, where it significantly contributes to the overall fertility and health of terrestrial ecosystems. This essential component, which is the seventh most common element in the Earth's crust, is necessary for the multiple functions that control the growth, metabolism, and developmental pathways of many plant species. Beyond merely facilitating plant metabolic activities, potassium plays a pivotal role in enhancing the quality of agricultural crops by aiding in the processes of grain filling and the augmentation of kernel weight, fortifying the structural integrity of plant straw, bolstering disease resistance against a plethora of pests and pathogens, and enabling plants to endure and thrive under conditions of environmental stress (Rawat, 2016). Furthermore, potassium is instrumental in activating a range of plant enzymes, enhancing the process of photosynthesis, reducing the rate of respiration, maintaining the essential turgidity of plant cells, facilitating the transport of carbohydrates such as sugars and starches, assisting in the uptake of nitrogen, and is equally vital for the synthesis of proteins necessary for plant growth and development (Prajapati, 2012). In the absence of sufficient potassium, plants are likely to exhibit poorly developed root systems, experience stunted growth, yield smaller seeds, and ultimately produce lower overall agricultural yields (Sustr, 2019). It is noteworthy that soils characterized by a deficiency of potassium are typically found to possess sandy textures, exhibit conditions of water logging, demonstrate salinity issues, and present a degree of acidity that further complicates agricultural practices. The limited availability of potassium in substantial regions of cultivated soils located in countries such as India, Korea, and China is a contributing factor to the observed decline in

crop productivity, underscoring the significance of potassium in sustainable agricultural practices (Basak, 2022).

Potassium-solubilizing bacteria, which are classified as heterotrophic microorganisms, derive their energy and cellular carbon from organic materials that already exist within their environment, thereby playing a crucial role in the formation of humus in soil ecosystems (Meena, 2016). In this context, these bacteria contribute not only to the cycling of essential minerals that are locked within organic matter but also exhibit the capability to solubilize potassium-bearing rock mineral powders, including but not limited to micas, illite, and orthoclase (feldspar), while simultaneously producing and excreting a variety of organic acids that facilitate these processes (Sindhu, 2016).

Production of carboxylic acids, such as citric, tartaric, and oxalic acids, has been documented as a primary mechanism that underpins the solubilization of potassium, demonstrating the biochemical strategies employed by these microorganisms (Li, 2020). The operational mechanism of potassium-solubilizing silicate bacteria is characterized by their ability to dissolve potassium, silicon, and aluminum ions from insoluble potassium-bearing minerals, including micas, illite, and orthoclase, through the excretion of organic acids that not only dissolve rock potassium directly but also chelates silicon ions, thereby enabling potassium to enter the solution and become bioavailable for plant uptake (Sharma, 2016).

10. Zinc Solubilizing biofertilizers:

Zinc, which is widely recognized as one of the most crucial micronutrients essential for the optimal growth and development of plants, plays a significant role in enhancing disease defense mechanisms and providing resistance against various forms of environmental stressors, as articulated in the scholarly work of (Sindhu et al. 2019). The deficiency of zinc in agricultural settings adversely impacts both the yields of crops and the nutritional quality of the produce, consequently resulting in a decline in the overall quality of crop products and giving rise to serious nutritional and health-related issues in human populations, particularly among vulnerable groups such as children. The element Zn is fundamentally indispensable in numerous physiological processes, including but not limited to the structural composition of a wide array of proteins, the maintenance of the structural and functional integrity of cellular membranes, the development of chloroplasts, and serving as a cofactor for a diverse range of enzymes, as well as being involved in critical biochemical and metabolic

processes such as photosynthesis, carbohydrate and auxin metabolism, nodulation, protein synthesis, and the metabolism of lipids and nucleic acids, as discussed in the literature by (Bhatt and Maheshwari 2020) and (Karnwal 2021a).

Zinc deficiency is particularly pronounced in calcareous, alkaline, and sodic soils that are characterized by low organic matter content combined with high pH levels, which collectively contribute to the desiccation of the uppermost layers of the soil surface. The availability of Zn is significantly influenced by the pH of the soil, primarily due to the fact that this essential element tends to be readily adsorbed onto cation exchange sites when the pH exceeds neutral levels, while becoming more accessible and available to plants when the pH is reduced to lower values, as noted by (Broadley et al. 2007). The amount of zinc that is accessible to plants is regulated by various soil factors, which include total zinc concentration, organic matter content, pH levels, clay composition, redox conditions, the presence of calcium carbonate, microbial activity in the rhizosphere, soil moisture, concentrations of other trace elements, and levels of macronutrients, particularly those related to climate and phosphorus, as highlighted by (Alloway, 2008).

Though the deficiency of Zn in crops can be effectively mitigated through the application of Zn-based chemical fertilizers in agricultural fields, the extensive utilization of these fertilizers frequently results in detrimental environmental consequences. As a viable alternative, numerous plant growth-promoting rhizobacterial (PGPR) strains have been recognized as highly effective agents that can significantly enhance the solubility of zinc in the soil. Zinc-solubilizing microbes (ZnSMs) are specialized microorganisms endowed with the capability to transform insoluble forms of zinc (Zn) present in the soil into soluble forms by synthesizing organic acids and enzymes that facilitate the degradation of insoluble zinc compounds found in the soil matrix. These beneficial bacteria colonize the rhizosphere and play a crucial role in converting complex insoluble zinc compounds into forms that are more readily accessible to plants, thereby contributing positively to enhanced plant growth and developmental processes, as noted by (Kushwaha et al. 2020). The solubilization of these complex compounds is achieved through a variety of mechanisms, which include the production of organic acids as described by (Vidyashree et al. 2018), the generation of chelated ligands as detailed by Singh and (Prasanna, 2020), the release

of protons, and the engagement of oxido-reductive systems at the cellular membrane level, as documented by (Upadhayay et al. 2022b). Therefore, it is of paramount importance for both farmers and researchers to consider the utilization of zinc-solubilizing microbes as biofertilizers or soil inoculants to enhance the uptake of zinc by crops, which can ultimately lead to improvements in plant growth, increased crop yields, and the overall health of the soil ecosystem while simultaneously reducing the reliance on chemical zinc fertilizers.

MATERIALS AND METHODS

➢ **Bacterial Cultures:** Different bacterial culture samples were supplied from the culture collection unit of Molecular and Applied Mycology and Plant Pathology Research Laboratory, Department of Botany, University of Calcutta. The culture samples were maintained in nutrient agar medium and sub cultured at regular interval. Additional investigations were conducted using these cultures.

- **Primary screening to detect special activity:**

  ◆ **Zinc Solubilizing test :** Petri plates were prepared with zinc solubilizing agar media. The bacterial samples were spot inoculated into the plates with the help of a sterile inoculating loop (Saravanan et al., 2003). The plates were incubated for 72 hours at 37°C. A halo zone surrounding the bacterial colonies indicates the zinc solubilizing property of the sample cultures.

  ◆ **Potassium Solubilizing Test :** Aleksandrov agar medium was prepared and the sample bacteria were spot inoculated into the plates with the help of a sterile inoculating loop, the plates were incubated for 72 hours at 37°C. A halo zone surrounding the bacterial sample colonies (M Saha, 2016) indicates the potassium solubilizing property of the sample cultures.

  ◆ **Phosphate Solubilizing Test:** Bacterial isolates were spot inoculated into the Pikovoskay's agar plates with the help of a sterile inoculating loop, the plates were incubated for 72 hours at 37°C. The presence of halo zone surrounding the bacterial colonies (Oliviera, 2009) indicates the phosphate solubilizing property of the sample cultures.

  ◆ **Nitrogen Fixation Test :** Petri plates were prepared with Jensen's Medium containing sucrose (20.0 g), K_2HPO_4 (1.0 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), NaCl (0.5 g), $FeSO_4 \cdot 7H_2O$ (0.1 g), $Na_2MoO_4 \cdot 2H_2O$ (0.005 g), and agar (20.0 g) and distilled water 1 L. All the bacterial samples were inoculated in the medium with the help of a sterile inoculating loop and were incubated in 27°C for 4-5 days (Ahmad, 2008). Growth of colonies in the media suggests that the bacterial samples were able to fix nitrogen as the media was nitrogen free. This indicates the nitrogen fixing ability of the bacteria in this nitrogen free medium.

- **Plant growth promoting activity**

  - **Detection of IAA production:** The procedure described by Brick et al. (1991) was employed to evaluate IAA production. The sample isolates were inoculated in Luria-Bertani (LB) broth supplemented with 50 µgml' of L-tryptophan, 100 µl of 10 mM O-phosphoric acid, and then incubated at 37°C while being shaken continiously. After incubation 4 ml of Salkowaski reagent (1 ml of 0.5 mM $FeCl_3$, in 35% HCIO) were then added concomitantly within 2 ml supernatant of the isolated bacterial cultures and observed for the formation of pink coloration of the broth which proved the IAA production capacity of the isolates.

  - **Detection of ammonia production:** In peptone water medium (Dye et al. 1962),The ability of the bacterial isolates to produce ammonia was assessed. The sample inoculum was inoculated from 24-hour-old agar slants and incubated for four days at 30°C. When 1 ml of Nessler's reagent was added, the colour changed from brown to yellow, confirming the isolates ability to produce ammonia (Cappucino and Sherman, 1992).

- **Characterization of Bacterial Samples:**

- **Morphological and Cultural Characterisation:** Gram and morphological characterisations were performed on young bacterial cultures (18–24 hours old).

  - **Gram Staining :** A bacterial smear was created by aseptically transferring a drop of bacterial suspension onto a clear, grease-free slide, drying it, and then heat-fixing it. For one minute, the fixed smear was coated with crystal violet solution. After that, iodine solution was applied to the slide for one minute. The iodine solution is washed away with absolute alcohol until colour ceases to come out of preparation. Then safranin was put on the slide to counterstain for 1 min, after that the slide was rinsed with water and dried with blotting paper to observe under the microscope (Skerman, 1967).
  - **Shape and arrangement:** The slide with the pigmented bacterial smear was lightly coated with a drop of immersion oil. The slide was then examined under a 100X Oil immersion objective lens of a compound microscope. The shape, arrangement and other morphological characteristic were noted.

- **Cultural Characteristics:** Different cultural parameters like motility, growth form, elevation, colour, surface edges etc. were tested in nutrient agar slants, plates and motility agar after incubation at 28°C for 48-72 hours .
- **Slant characters:** Different bacterial culture samples were grown on nutrient agar slants and incubated at 28± 2°C. There cultural characteristics were recorded as;

  Growth: Scanty, moderate, abundant

  Surface: Smooth, rough, dry, moist
- **Plate characters:** Different bacterial culture samples were grown on Nutrient agar plates and incubated at 28± 2°C. There cultural characteristics were recorded as;

  Growth: Slow, moderate, rapid

  Form: Punctiform, Circular, rhizoid, irregular, round

  Surface: Smooth, rough, dry, moist

  Margin: Entire, Lobate, Filifrom, Serrate, Undulate

  Elevation: Flat, Raised, Convex

- **Capsule Staining:** A small amount of nigrosine dye was added to a clear, grease-free slide. After that a small quantity of bacterial culture was added to the dye aseptically. Another slide was used to generate a smudge. After being allowed to air dry, the smear was examined under a microscope.While the bacteria itself gets stained, the capsule remains untreated. The capsule appears as faint and light coloured surrounding the stained bacteria. The method of (Breakwell, 2009) was followed.

- **Endospore Staining:** A drop of bacterial suspension was aseptically transferred on a clear grease free slide and bacterial smear was made. The smear was then air dried to fix. The fixed smear was then placed over a beaker containing water on simmering boil. The smear was then covered with 5% malachite green and kept moist for 10 mins. The excess stain was then rinsed with distilled water. After 5 minutes of safranin flooding, the smear was washed with distilled water, air dried, and was examined under a microscope. The method of (Hussey, 2007) was followed.

- **Biochemical Characterization**

  - **Indole Test:** Prepare tryptophan broth (tryptophan-10 gm, water-1 lit.) and inoculate it with bacterial culture and then incubate the culture 37°c för 48 hours. Add 0.5 ml of the Kovac's reagent (p-dimthylbenzaldehyde-15 gm, isoamyl alcohol-150 gm, HCL-75 gm.) after the bacterial growth. After 2 minutes a red colour may appear at the junction of medium. On other hand indole is diffused above the medium and paper strip turns pink if the strip is already impregnated with oxalic acid (Dubey and Muheshwari, 2002).

$$\text{Tryptophan} + H_2O \xrightarrow{\text{Tryptophanase}} \text{Indole} + \text{Pyruvic Acid} + H_2O$$

  - **Methyl red test**: The ability of different isolates to produce acid from glucose in amounts sufficient to reduce the pH to 4.2 or less and to maintain this low pH for at least 4 days was determined by using the glucose-phosphate-peptone-water method outlined by Skerman (Skerman, 1967). Cultures were tested for acid production 2 and 4 days after inoculation by adding a few drops of methyl red solution. The presence of red colour was taken as a positive indication and yellow colour as a negative indication.

$$\underset{\text{(pH 6.0)}}{\text{Methyl Red (yellow colour)}} \longrightarrow \underset{\text{(pH 4.4 or less)}}{\text{Methyl Red (Red colour)}}$$

$$\alpha\text{-D-Glucose} \longrightarrow \text{pyruvic acid} \longrightarrow \text{mixed acid } (\downarrow \text{pH}) + CO_2$$

  - **Voges-Proskauer test**: The ability of different isolates to produce acid from glucose and subsequently to convert it to acetylmethylcarbinol or 2:3-butylene glycol, both neutral substances, was determined by the method outlined by Skerman (Skerman, 1967). Cultures grown in glucose-phosphate-peptone-water were tested for the presence of a neutral substance with 1ml of 10% KOH solution, 2 and 4 days after incubation. The presence of pink colour was scored positive and no colouration, negative. According to the method outlined by Shaffer (1967) using phenol red. Cultures were observed for acid and gas production after two days.

Glucose ⟶ Pyruvate ⟶ Acetoin (Intermediate product)

Acetoin $\xrightarrow{O_2, KOH}$ Diacetyl + H_2O

Diacetyl + Guanidine component of peptone $\xrightarrow{\alpha\text{- napthol}}$ pink-red colour

◆ **Citrate utilisation test:** With the help of an inoculating loop a small amount of test culture was transferred into the test tubes containing Citrate Broth. For two to four days, the inoculation tubes were incubated. The broth was checked for colour and turbidity changes after incubation. A change in the broth's hue and turbidity signifies a successful test outcome. A shift in the colour and turbidity of the broth indicates a positive result of the test. A negative result is indicated by the lack of change in colour and turbidity of the broth Koser, 1924).

◆ **Detection of Cellulase Production**: For cellulase, petri plates with 1% (v/v) MSM fortified with 1% (w/v) carboxymethyl cellulose (Teather et al. 1982) were used to inoculate the samples and incubated for 48 hrs. After incubation the plates were then flooded with 0.1% aqueous solution of Congo red for 15 mins followed by repeated washing with 1 M NaCl. The colony of the samples producing visible halos were the proof of their extra-cellular cellulase production capacity.

◆ **Detection of Amylase Production:** Plates of nutrient agar containing 1.0% soluble starch were inoculated with culture samples and incubated at 28°c. Iodine solution was added to the plates after 48 hours, and they were left for 10 to 15 minutes. The iodine solution was then removed by washing. The bacterial growth was surrounded by a clear region, which suggested starch hydrolysis (Shinde and Lukezic, 1974).

◆ **Carbohydrate utilisation test :** The ability of bacterial isolates to ferment various carbohydrates with acid or gas production were tested in tryptone media by following the method of (Sneath et al., 1986), (Shukla et al., 2014) and (Cappuccino and Welsh 2017). 1gm Tryptone media with (0.5gm Dextrose/ Fructose/ Arabinose/ Sucrose/ Lactose), 0.5gm NaCl and 0.018 gm

phenol red was dissolved in 100ml Distilled water. In each test tubes inverted sterile Durham's tubes were carefully added without incorporation of bubble and autoclaved. Isolated samples were then Inoculated in the media and were incubated for 72hrs. After incubation a change in colouration from red to yellow and bubble formation inside the Durham's were inspected. The bacteria that fermented the carbohydrate changed the pH of the media into acidic.The phenol red indicator changed from red to yellow as the media became more acidic.

- **Urease test :** The ability of bacterial sample isolates to hydrolyze urea and produce ammonia and carbon dioxide was tested in Urea broth by following the method of (Brink, 2010).Phenol red, a pH indicator, and 2% urea are present in urease test media. An increase in pH due to the production of ammonia results in a color change to bright pink (pH 8.2).

- **Triple sugar iron agar test:** To test Triple Sugar Iron Agar fermentation by bacterial sample isolates, the method outlined by (Lehman, 2005) agar slants containing Tryptone powder (10 g), Peptone powder (10 g), Beef extract (3 g), yeast extract (3 g), Lactose (10 g), Sucrose (10 g), Glucose (1 g), NaCl (5 g), $FeSO_4$ (0.2 g), Sodium Thiosulfate (0.3g), Phenol Red (0.024g), agar powder (13 g) in g/L composition was prepared. A loopful of isolate from freshly subcultured slants were taken and stabbed in the but region of the tube

- **ISOLATION OF GENOMIC DNA :** For Isolation of genomic DNA of all the sample isolates (HiGenoMB -MB505) Kit was used. The isolation protocol provided in the bacterial Genomic DNA Isolation kit was followed:

Step 1: 1.5 ml of bacterial broth culture were centrifuged at 13,000 rpm for 2 mins. The culture cells were pelleted and the culture medium were completely discarded. **Step 2:** The pellet was then resuspended in 200 µl of lysozyme solution (45 mg/ml) and incubated for 30 mins at 37°C .

Step 3: 20 µl of Proteinase K solution (20 mg/ml) was added along with 20µl of RNase A solution. Incubated for 5 mins at 25°C.

Step 4: 200 µl of Lysis Solution was added. Vortexed thoroughly for few seconds then incubated at 55°C for 10 mins.

Step 5: 200 µl of ethanol (95-100%) was added to the lysate and vortexed thoroughly.

Step 6: The Lysate was then transferred into the Spin Column and centrifuged at 10,000 rpm for 1 min at room temperature. The flow through liquid was then discarded and the spin column was placed in the same 2 ml collection tube.

Step 7: 500 µl of Prewash solution was added to the column and centrifuged at 10,000 rpm for 1 min at room temperature. The flow through liquid was then discarded and the spin column was placed in the same 2ml collection tube.

Step 8: After adding 500 µl of diluted Wash Solution to the spin column, it was centrifuged for three minutes at room temperature at 13,000 rpm. After discarding the flow-through liquid, the spin column was centrifuged once more for one minute at 13,000 rpm in order to dry it out.

Step 9: The Spin column was then Transferred to a fresh 2 ml collection tube. 200 µl of Elution Buffer was then added to the spin column and cetrifuged at 10,000 rpm for 1 min at room temperature to elute the DNA.

Step 10: The elute (Isolated Genomic DNA) was then transferred to a fresh capped 2 ml collection tube for storage.

- **PCR amplification :** All of the isolated genomic DNA of the bacterial samples were then amplified in the thermal cycler along with PCR master-mix (10X Buffer 2.5 µl , dNTP 0.5 µl, Forward primer 0.5 µl, Reverse primer 0.5 µl, Taq DNA polymerase 0.25 µl, $MgCl_2$ 0.75 µl, H_2O 19 µl, DNA template 1 µl) (Mehnaz, 2001).
- **Primers Used for amplification** : Forward primer sequence " 27F (5'-AGAGTTTGATCCTGGCTCAG-3') " , Reverse Primer sequence " 1492R (5'-GGTTACCTTGTTACGACTT-3') " was used.

RESULTS

Ten bacteria B44, B45, B47, B54, B55, B57, B60, B67, B68, B80 were isolated previously from soil from different places of West Bengal and their morphological, cultural, physiological and biochemical parameters were evaluated. These 10 different bacterial isolates were screened according to their plant growth promoting activities like zinc solubilizing, potassium solubilizing, Phosphate solubilizing and Nitrogen fixation ability.

- **Screening for PGPR activity**

  - **Zinc Solubilization** : Zinc solubilizing activity was tested for all the ten different isolated samples as described in the materials and methods. Out of the ten isolated samples only B60 showed (Fig-1). The results were recorded as high (+++), moderate (++), low (++) and Negative (-). (Table 1)

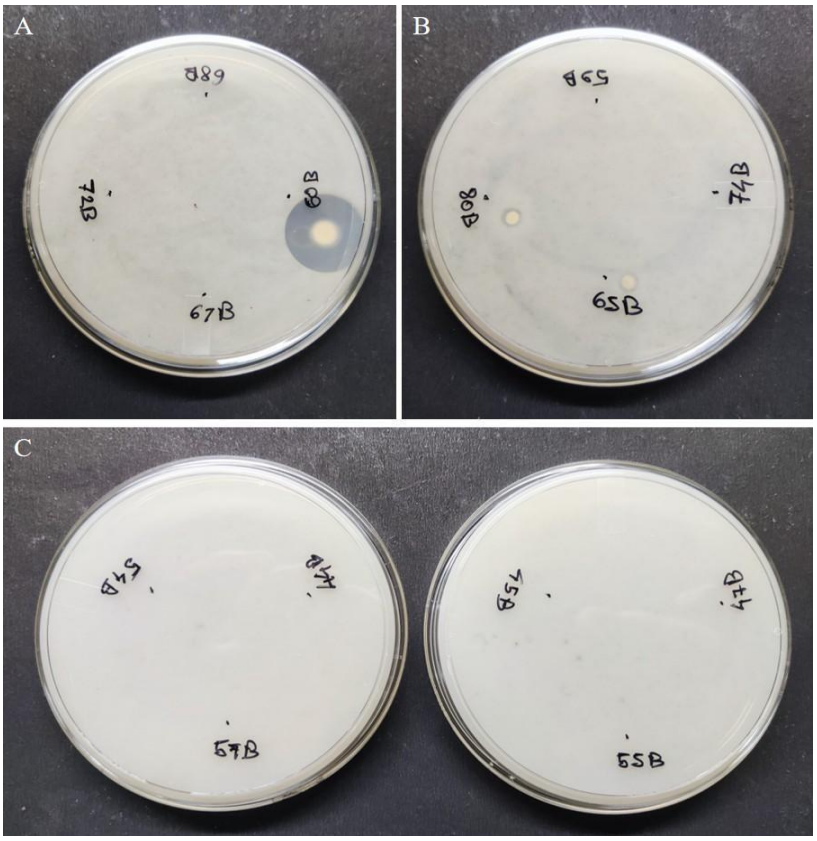

Fig 1: A- Represents Zinc Solubilizing activity plate of B60, B67, B68. B- Represents Zinc Solubilizing activity plate of B80. C-Represents Zinc Solubilizing activity plate of B44, B45, B47, B54, B55, B57.

- **Phosphate Solubilization** : Phosphate solubilizing activity was tested for all the ten different isolated samples as described in the materials and methods. Out of the ten isolated samples B44, B45, B47, B54, B55, B60, B68 showed phosphate solubilizing abilities (Fig - 2). The results were recorded as high (+++), moderate (++), low (++) and Negative (-) (Table 1)

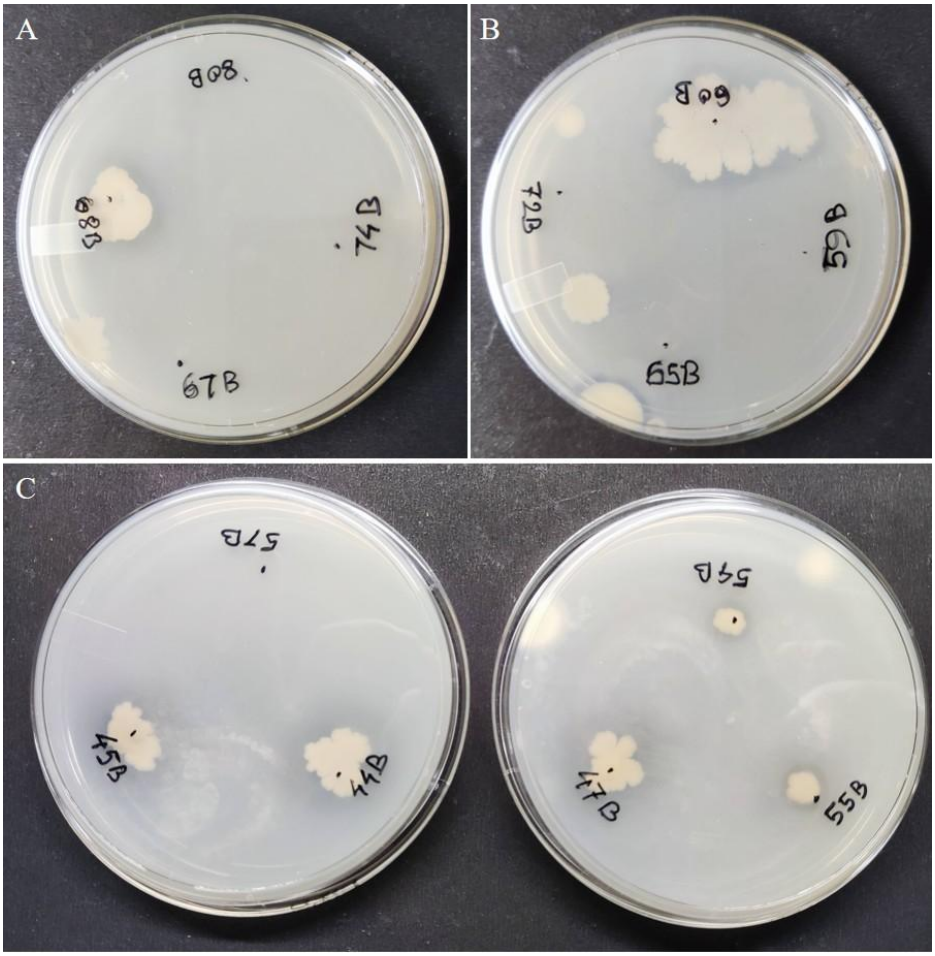

Fig 2: A- Represents Phosphate Solubilizing activity plate of B80, B67, B68. B- Represents Phosphate Solubilizing activity plate of B60. C-Represents Phosphate Solubilizing activity plate of B44, B45, B47, B54, B55, B57.

- **Potassium Solubilizing** : Potassium Solubilizing activity was tested for all the ten different isolated samples as described in the materials and methods. Out of the ten isolated samples, two (B60, B68) showed Potassium solubilizing abilities (Fig - 3). The results were recorded as high (+++), moderate (++), low (++) and Negative (-). (Table 1)

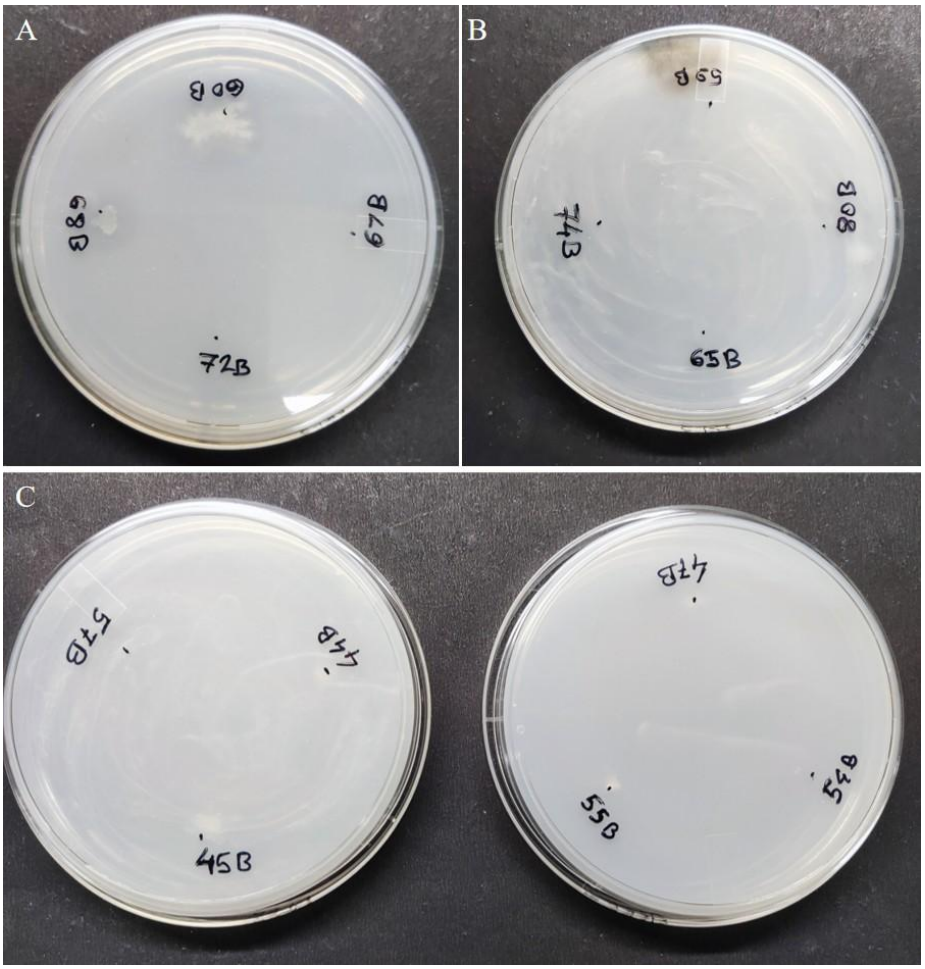

Fig 3: A- Represents Potassium Solubilizing activity plate of B60, B67, B68. B- Represents Potassium Solubilizing activity plate of B80. C-Represents Potassium Solubilizing activity plate of B44, B45, B47, B54, B55, B57.

- **Nitrogen Fixation Ability** : Nitrogen Fixation Ability was tested for all the ten different isolated samples as described in the materials and methods. Out of the ten isolated samples B44, B45, B47, B54, B55, B57, B60, B67, B68, B80 all of them showed Nitrogen Fixing abilities (fig-4). The results were recorded as high (+++), moderate (++), low (++) and Negative (-). (Table 1)

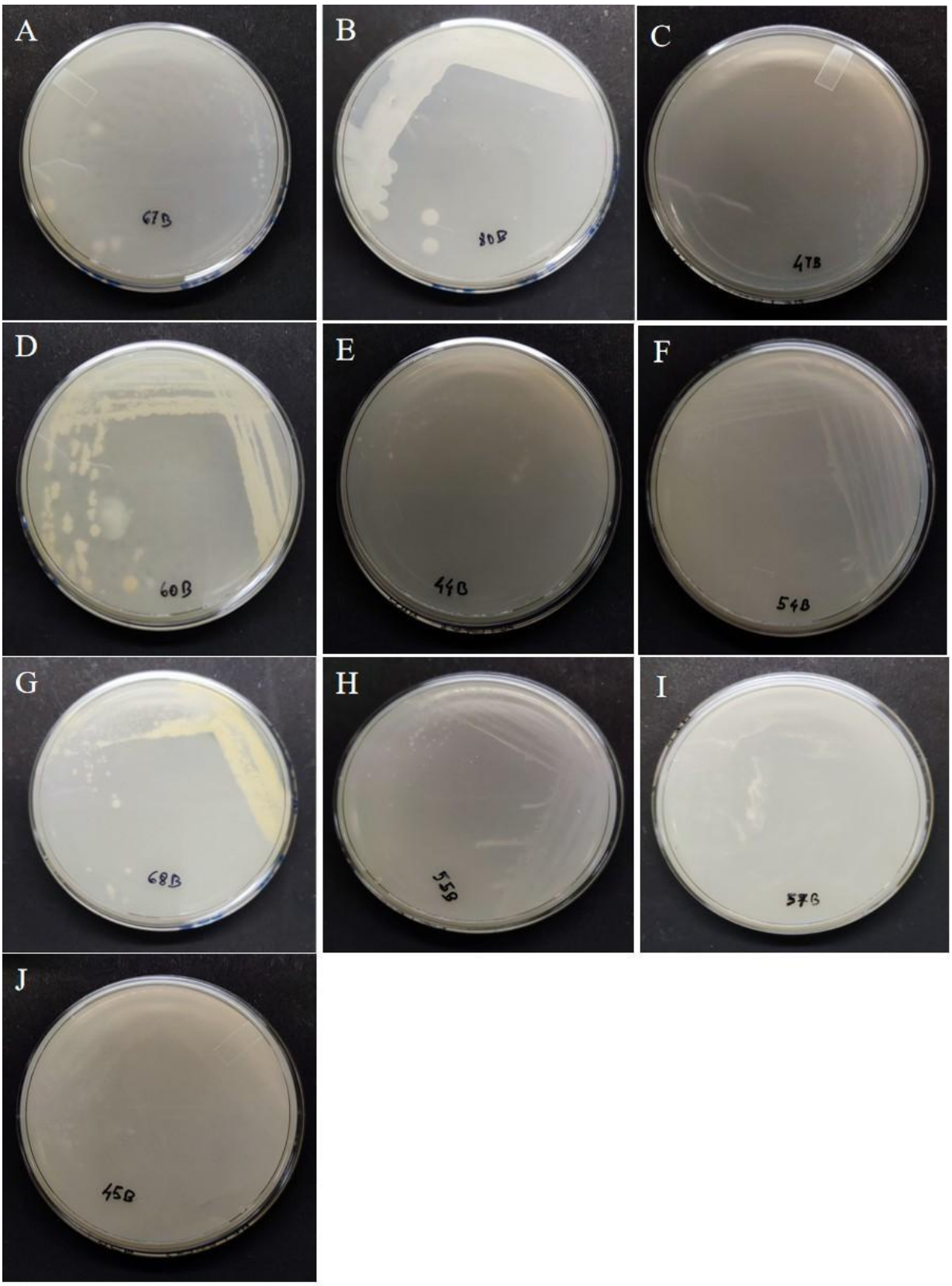

Fig 4: A-J Represents Nitrogen - Fixing ability test plates of all ten Bacterial Sample isolates.

- **IAA Production** : All the 10 bacterial isolates were tested for IAA Production according to the method outlined by (Brick et al. 1991) which was described earlier in the Materials and Methods section. Formation of pinkish colour after addition of 4 ml of Salkowaski reagent (1 ml of 0.5 mM FeCl, in 35% HCIO) into the obtained supernatant of the samples indicates Positive result of the IAA production test. All bacterial samples showed very faint pinkish color (Fig - 5). The results were recorded as positive (+) (Table 1)

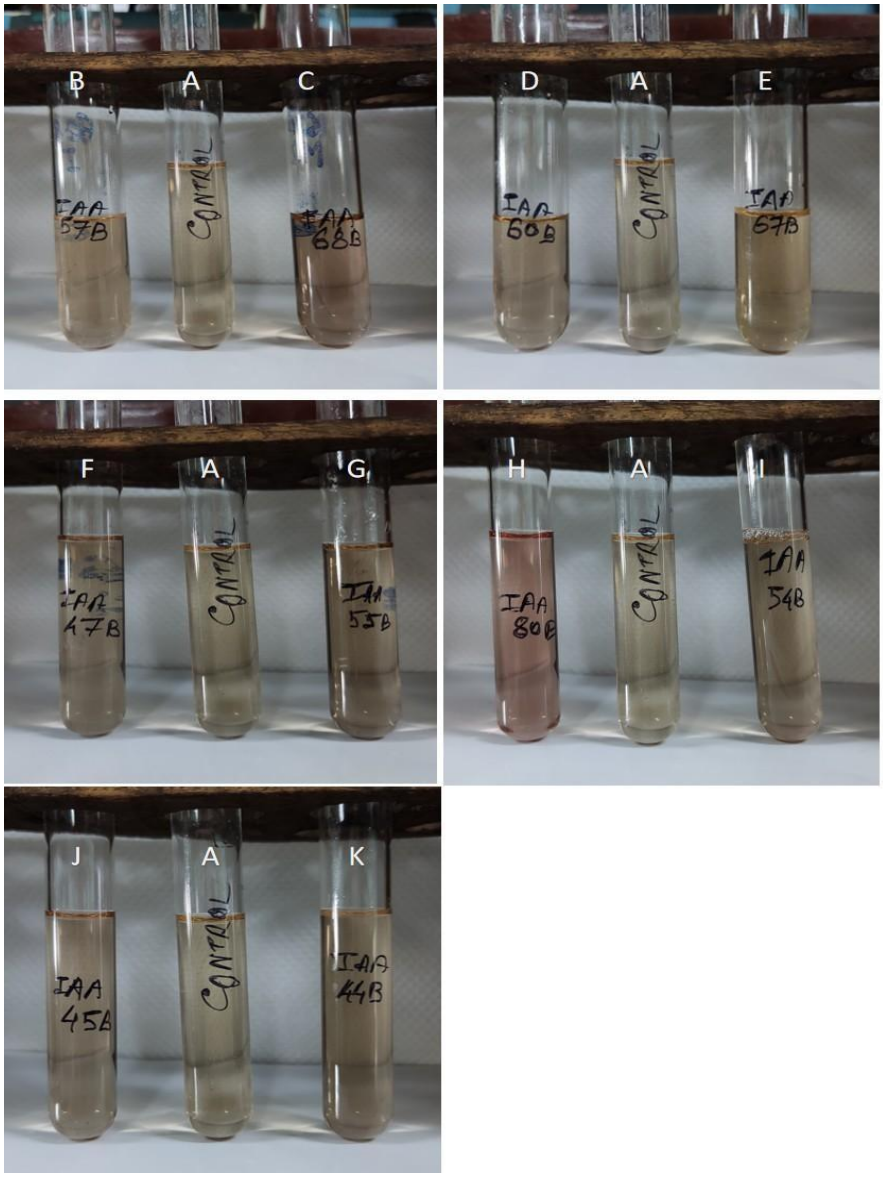

Fig 5 : A represents Control of the IAA production test, B-K represents sample isolates that produced IAA. (**B**- B57, **C**- B68, **D**- B60, **E**- B67, **F**- B47, **G**- B55, **H**- B80, **I**- B54, **J**- B45, **K**- B44)

- **Ammonia Production :** All the bacterial isolates were tested for production of ammonia according to the method outlined by (Dye et al. 1962 and Cappucino et al., 1992) which is mentioned earlier in the materials and methods section. All the bacterial samples formed yellow colour in the media after addition of 1 ml Nessler's reagent that confirmed the ammonia production capacity of the isolates (Fig - 6). The results were recorded as positive (+) (Table 1)

Fig 6 : A represents Control for the Ammonia production test. B-K represents all the ten sample isolates producing Ammonia

Table 1 : Plant Growth promoting activities exhibited by sample isolates

Sl No.	Identification No.	Phosphate Sb	Potassium Sb	Zinc Sb	Nitrogen Fixation	IAA Production	Ammonia Production
1	B44	+	—	—	+	+	+
2	B45	+	—	—	+	+	+
3	B47	+	—	—	+	+	+
4	B54	+	—	—	+	+	+
5	B55	+	—	—	+	+	+
6	B57	—	—	—	+	+	+
7	B60	+	+	+	+	+	+
8	B67	—	—	—	+	+	+
9	B68	+	+	—	+	+	+
10	B80	—	—	—	+	+	+

- **Morphological and cultural characterization :** All ten bacterial isolates were gram positive i.e the isolates retained the crystal violet stain and remained violet in colour. Most of the isolates were short rod shaped and few of them formed chains. B68 and B80 formed EPS. The form, colour, growth, elevation, margin of the isolated samples were recorded and are mentioned in (Table 2)

Table 2: Culture Characteristics and Colony morphology of sample isolates

Bacterial No.	shape	size	margin	elevation	form color	motility	pigmentation
B44	circular	small	entire	flat	milky white	—	creamy white
B45	circular	medium	entire	flat	milky white	—	creamy white
B47	circular	large	undulate	flat	milky white	—	creamy white
B54	circular	small	entire	flat	milky white	+	creamy white
B55	circular	small	entire	flat	milky white	+	creamy white
B57	circular	medium	entire	flat	milky white	—	dull white
B60	circular	medium	entire	flat	milky white	—	dull white
B67	circular	small	entire	flat	opaque	+	light yellow to white
B68	circular	medium	undulate	raised	milky white	+	dull white
B80	circular	small	entire	raised	milky white	—	dull white

- **Gram Staining** : Freshly subcultured sample isolates were Gram stained according to the method outlined by (Skerman, 1967), that was previously mentioned in the materials and methods section. Out of ten sample isolates all of them were Gram positive (Fig - 7). The results were recorded in Table-3 as positive (+) or negative (-).

- **Capsule Staining:** Freshly subcultured sample isolates were stained to check capsule formation according to the method outlined by (Breakwell, 2009), that was previously mentioned in the materials and methods section. Out of ten sample isolates all of them showed capsule formation (Fig - 8).The results were recorded in Table-3 as positive (+) or negative (-).

- **Endospore Staining:** Freshly subcultured sample isolates were stained to check endospore formation according to the method outlined by (Hussey, 2007), that was previously mentioned in the materials and methods section. Out of ten sample isolates only three of them (B44, B45, B60) showed endospore formation (Fig - 9).The results were recorded in Table-3 as positive (+) or negative (-).

Table 3: Microscopic characteristics of sample isolates

Bacterial No.	Gram Character	Endospore	Capsule	cell shape	cell arrangement
B44	+	+	+	med rod	palisade form
B45	+	+	+	long rod	In singles
B47	+	—	+	short rod	In singles
B54	+	—	+	med rod	Chain formation
B55	+	—	+	short rod	In singles
B57	+	—	+	med rod	In clumps
B60	+	+	+	long rod	In singles
B67	+	—	+	short rod	In singles
B68	+	—	+	short rod	In clusters
B80	+	—	+	short rod	few Chains

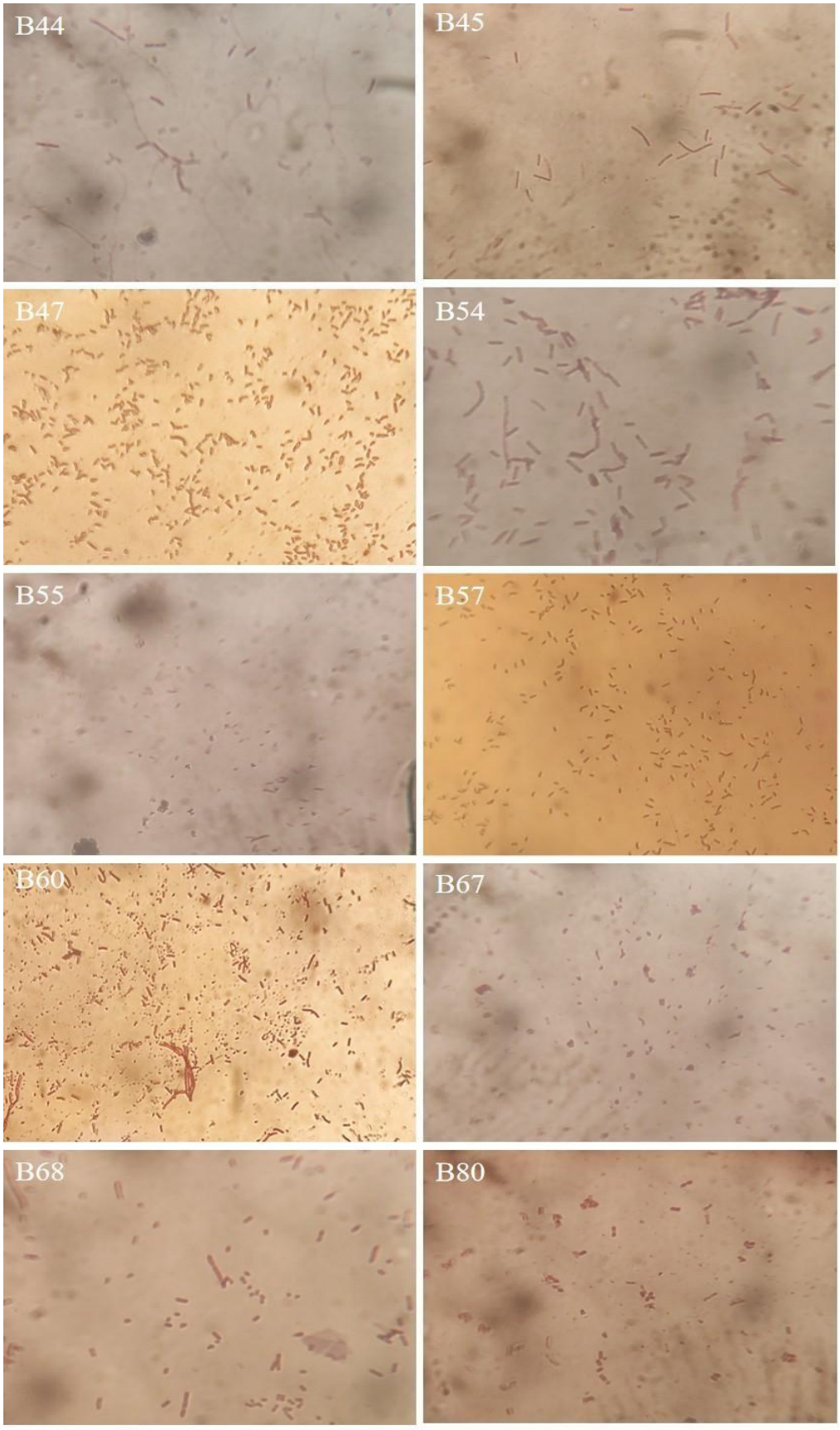

Fig 7 : Gram Characterization of sample isolates

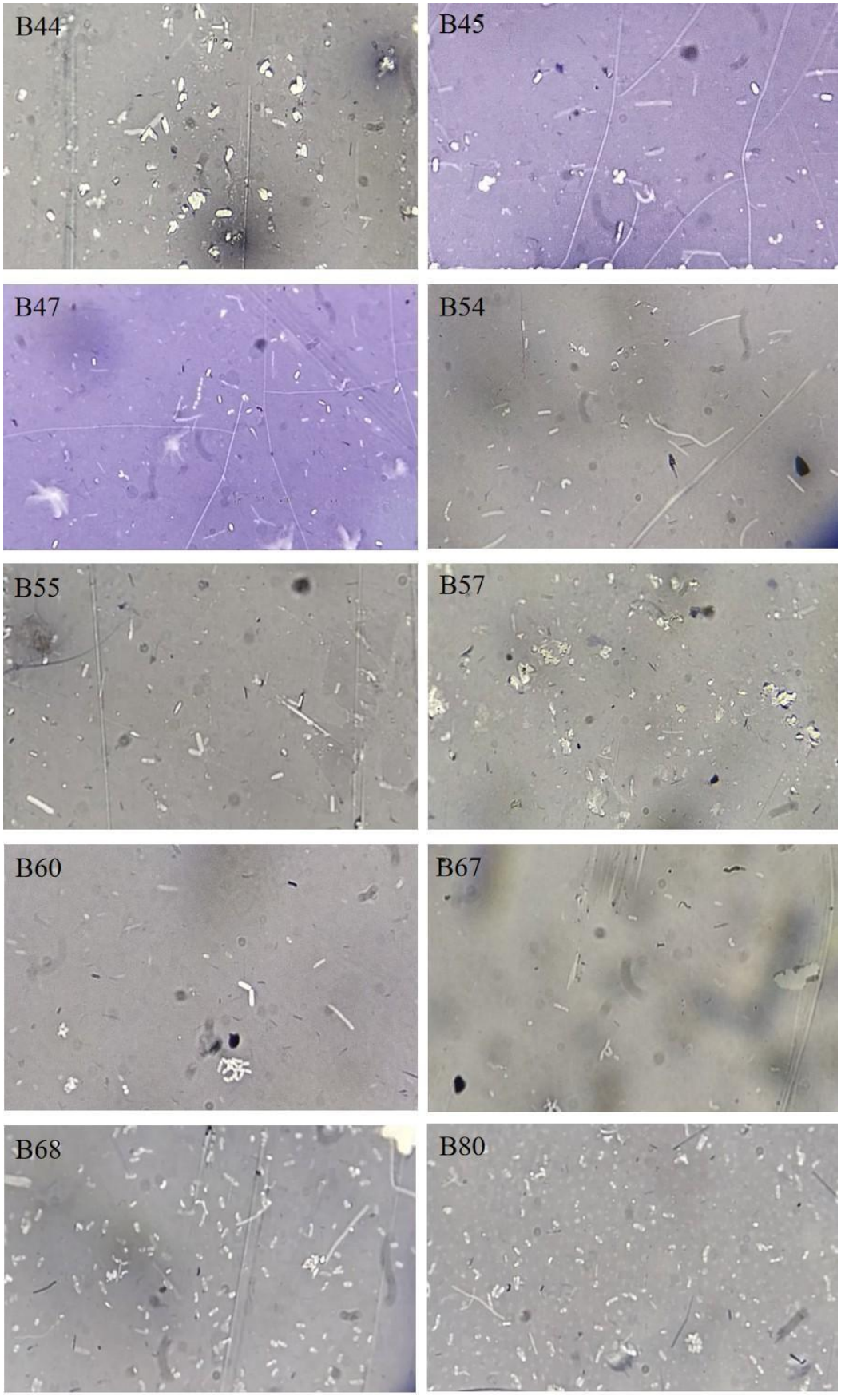

Fig 8 : Capsule stained sample isolates

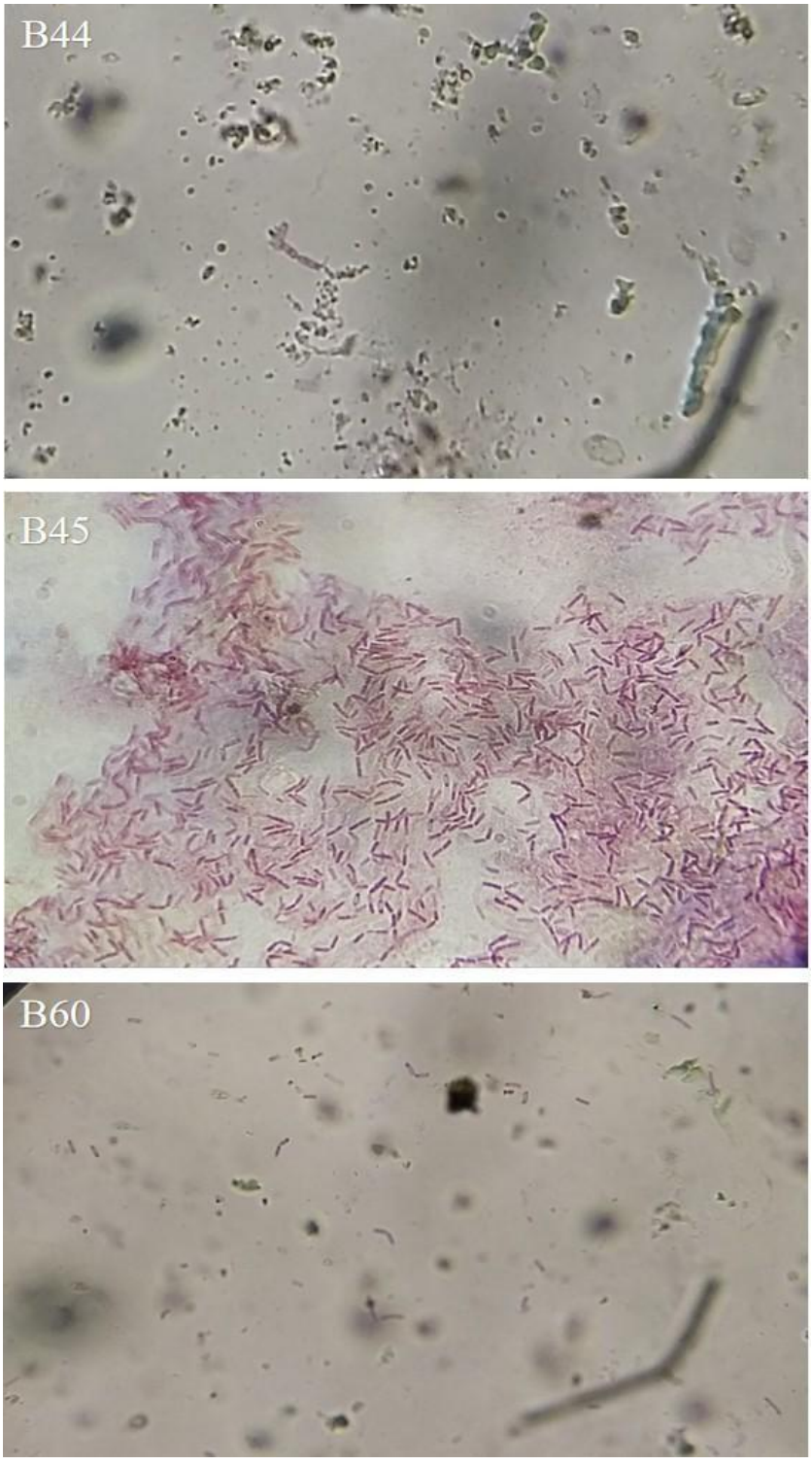

Fig 9 : Endospore stained sample isolates

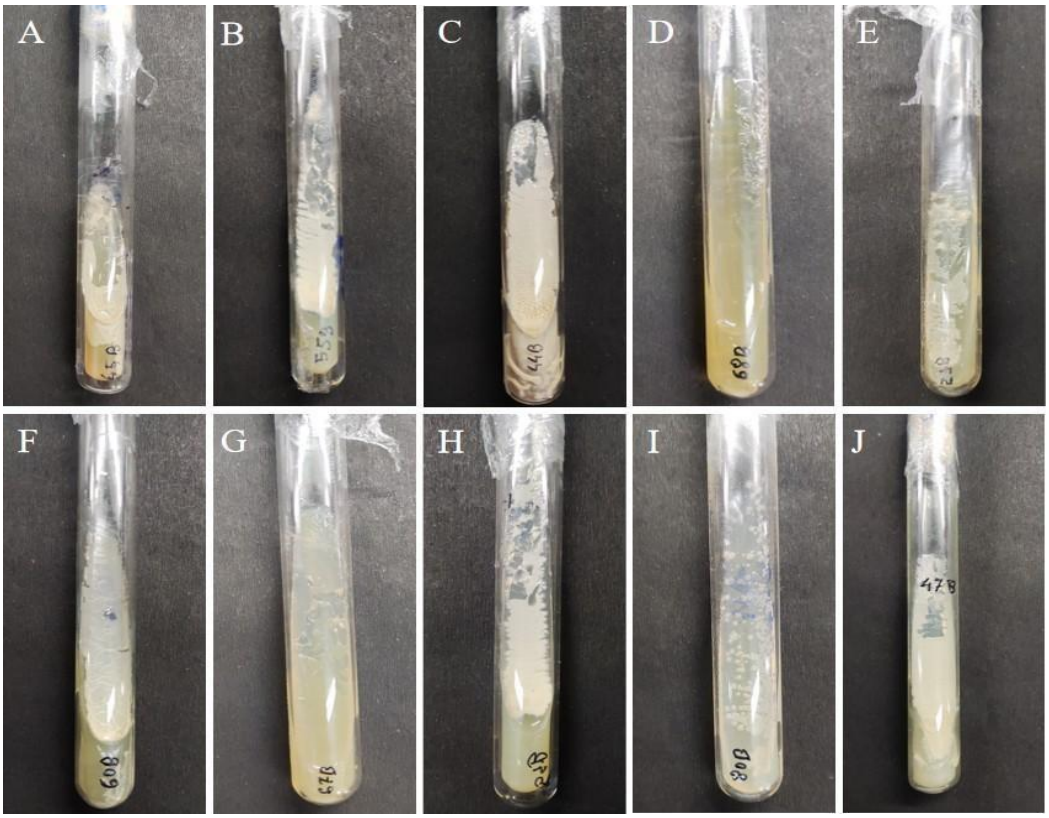

Fig 10 : Culture Slants of sample isolates

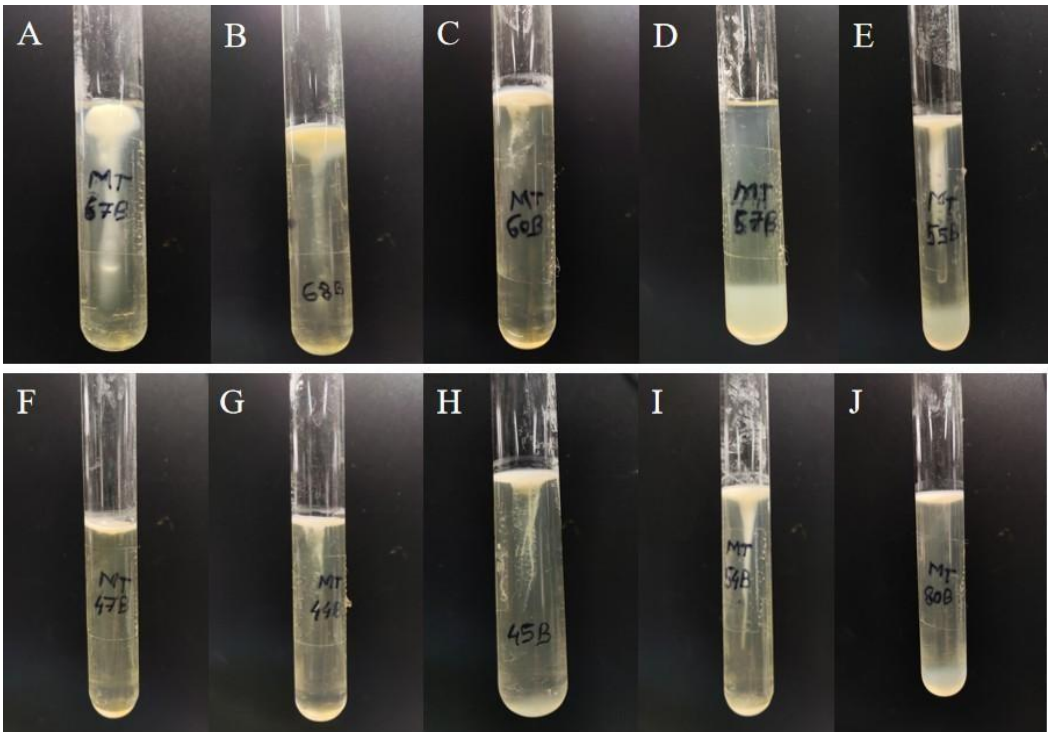

Fig 11 : A-J represents ten bacterial sample isolates in motility agar medium

- **Biochemical Characteristics**

◆ **Indole production :** All ten isolated samples were tested for Indole production by the method mentioned in the Materials and Methods section. Out of ten isolated samples no strains were able to change the colour of oxalic acid shoked filter paper to pink colour indicating indole negative result (Fig - 12). The results were recorded as positive (+) and negative (-) (Table 4).

◆ **Methyl Red Test :** Methyl red test was performed for all the isolates which is described details in materials and methods. After 2-4 days when the cultures were inoculated with Methyl red solution only two strains (B67 and B80) taken the red colour which indicate a positive reaction and most of the strains where negative because they took the yellow colour (Fig - 12). The results were recorded as positive (+) and negative (-) (Table 4).

◆ **Voges-Proskauer Test :** All the bacterial isolates were tested for Voges Proskauer test according to the method outlined by Skerman (1967), which is described earlier in the Materials and Methods section. When the cultures were treated with 10% KOH solution there were changes in colour into pinkish red indicating Voges-Proskauer test positive result (Fig - 12). The results were recorded as positive (+) and negative (-) (Table 4)

◆ **Citrate utilisation test :** All the sample isolates were tested for citrate utilisation by the method outlined by Koser (1924), which is mentioned in the Materials and Methods. After minimal inoculation with the sample cultures, all the sample isolates were able to turn the broth turbid indication a positive result of the Citrate utilisation test (Fig - 12). The results were recorded as positive (+) and negative (-) (Table 4).

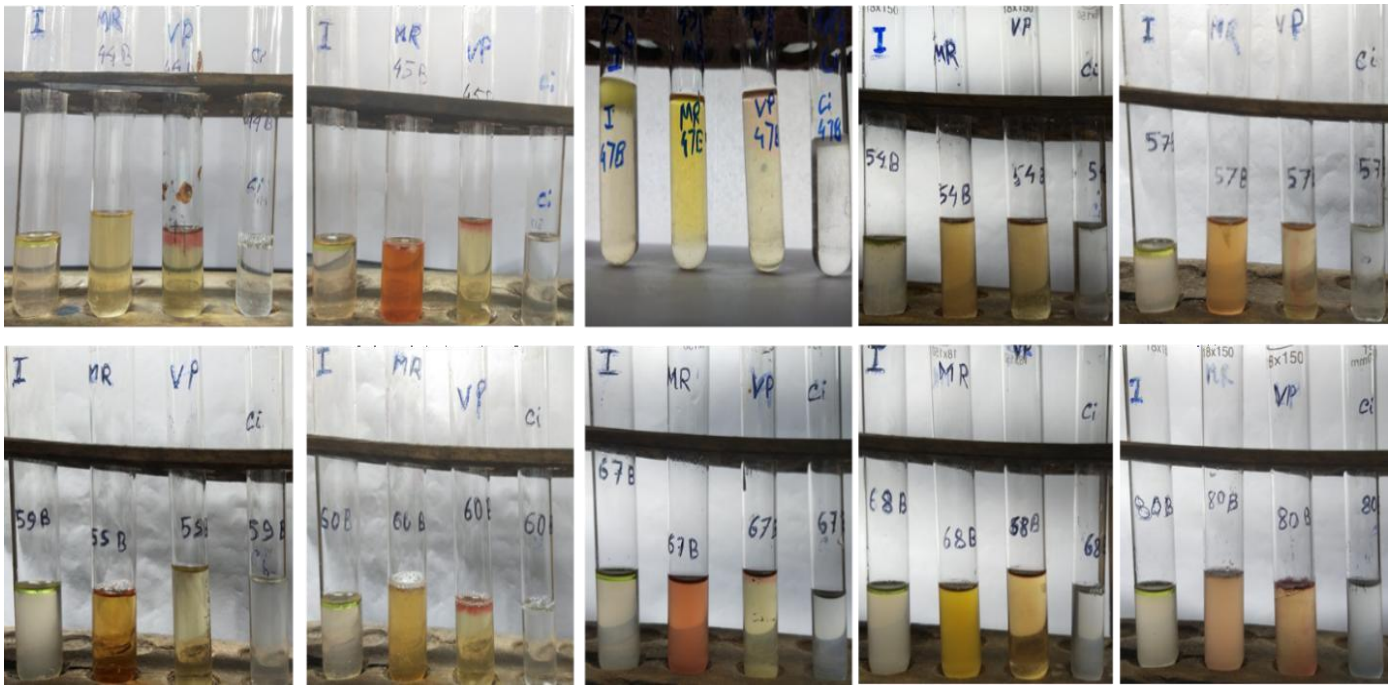

Fig 12 : IMViC test of ten bacterial isolates

◆ **Amylase activity**: Amylase activity of all the ten bacterial isolates were tested on 5X minimal media supplemented with 1% starch as described in materials and methods. After incubation for 24 h the plates were flooded with lodine solution, hydrolysis of starch showing a clear zone around the colonies. Out of ten bacterial samples B44, B45, B47, B60, B68 showed amylase activity (Fig - 13). The results were recorded as positive (+) and negative (-) (Table 4).

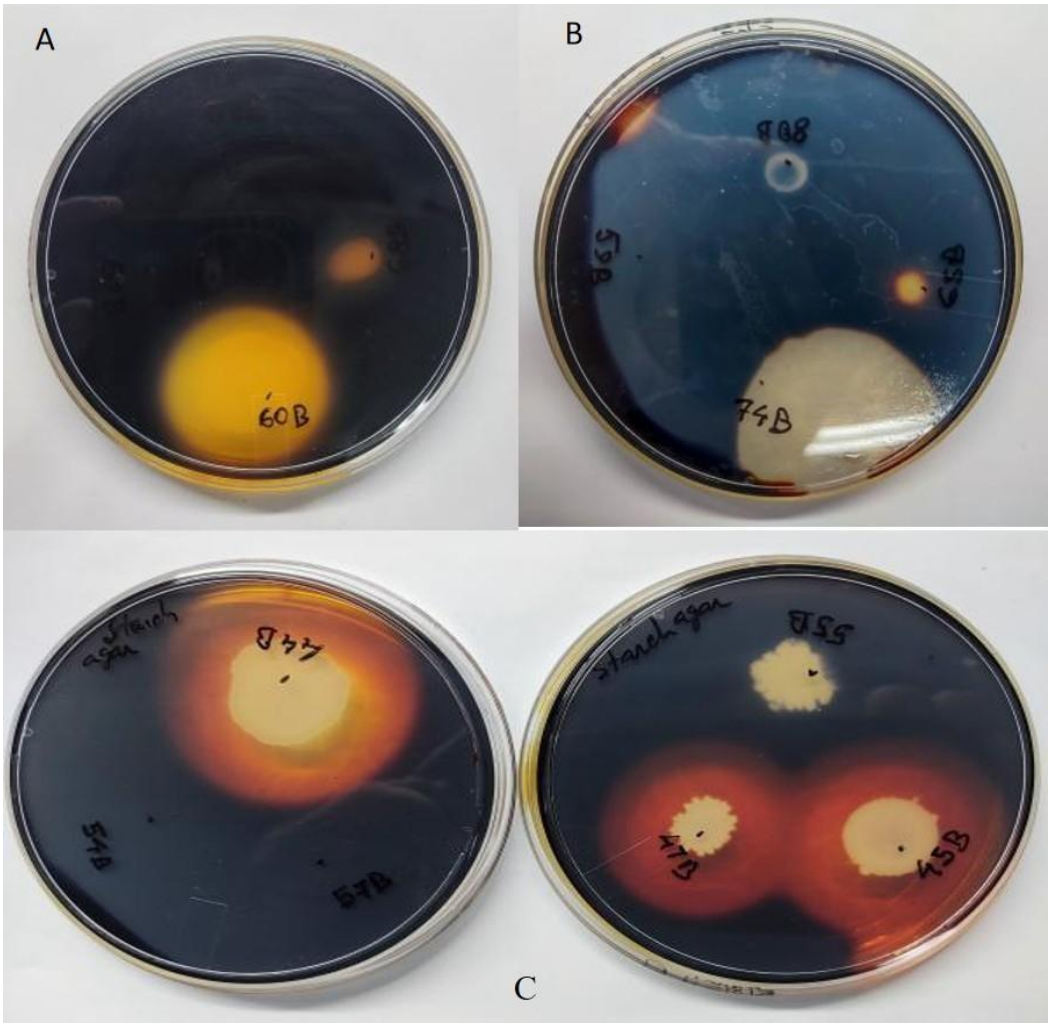

Fig 13: A- Represents Starch hydrolysis activity plate (Amylase test) of B60, B67, B68. B- Represents Starch hydrolysis activity activity plate of B80. C-Represents Starch hydrolysis activity activity plate of B44, B45, B47, B54, B55, B57.

◆ **Cellulose activity:** Cellulose activity of all the ten bacterial isolates were tested on minimal media supplemented with 1% Carboxy Methyl Cellulose (CMC) described in materials and methods. After incubation the plates were then flooded with 0.1% aqueous solution of Congo red for 15 mins followed by repeated washing with 1 M NaCl. The colony of the samples producing visible halos were indicating as positive result of the test. Out of ten bacterial samples B45, B47, B54, B55, B60 showed cellulose utilization (Fig - 14) The results were recorded as positive (+) and negative (-) (Table 4).

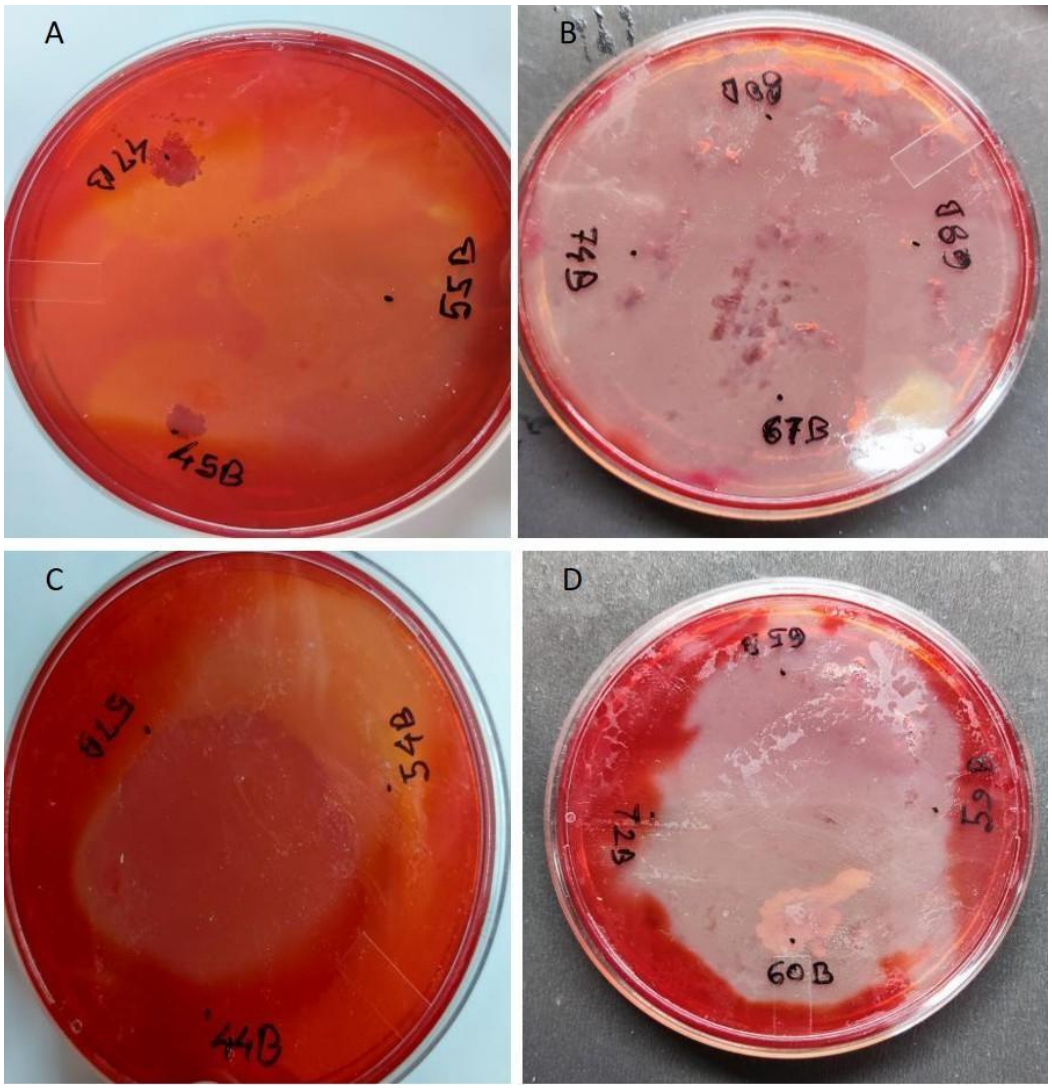

Fig 14: A- Represents Cellulose utilizing activity plate of B47, B45, B55. B-Represents Cellulose utilizing activity activity plate of B67, B68, B80. C-Represents Cellulose utilizing activity plate of B44, B54, B57. D-Represents Cellulose utilizing activity plate of B60.

◆ **Carbohydrate utilisation Test :** All the sample isolates were tested for different carbohydrate utilisation by the method outlined by (Sneath et al., 1986), (Shukla et al., 2014) and (Cappuccino and Welsh 2017), which is mentioned earlier in the Materials and Methods. All the bacterial isolates were incubated in Tryptone based media with Phenol Red Indicator and inverted Durham's tube . A bubble inside the Durham's Tube showed the formation of gas, and the colouration changed from red to yellow, indicating a successful outcome and proof of the fermentation of carbohydrates (Fig - 15). The results were recorded as positive (+) and negative (-) (Table 4).

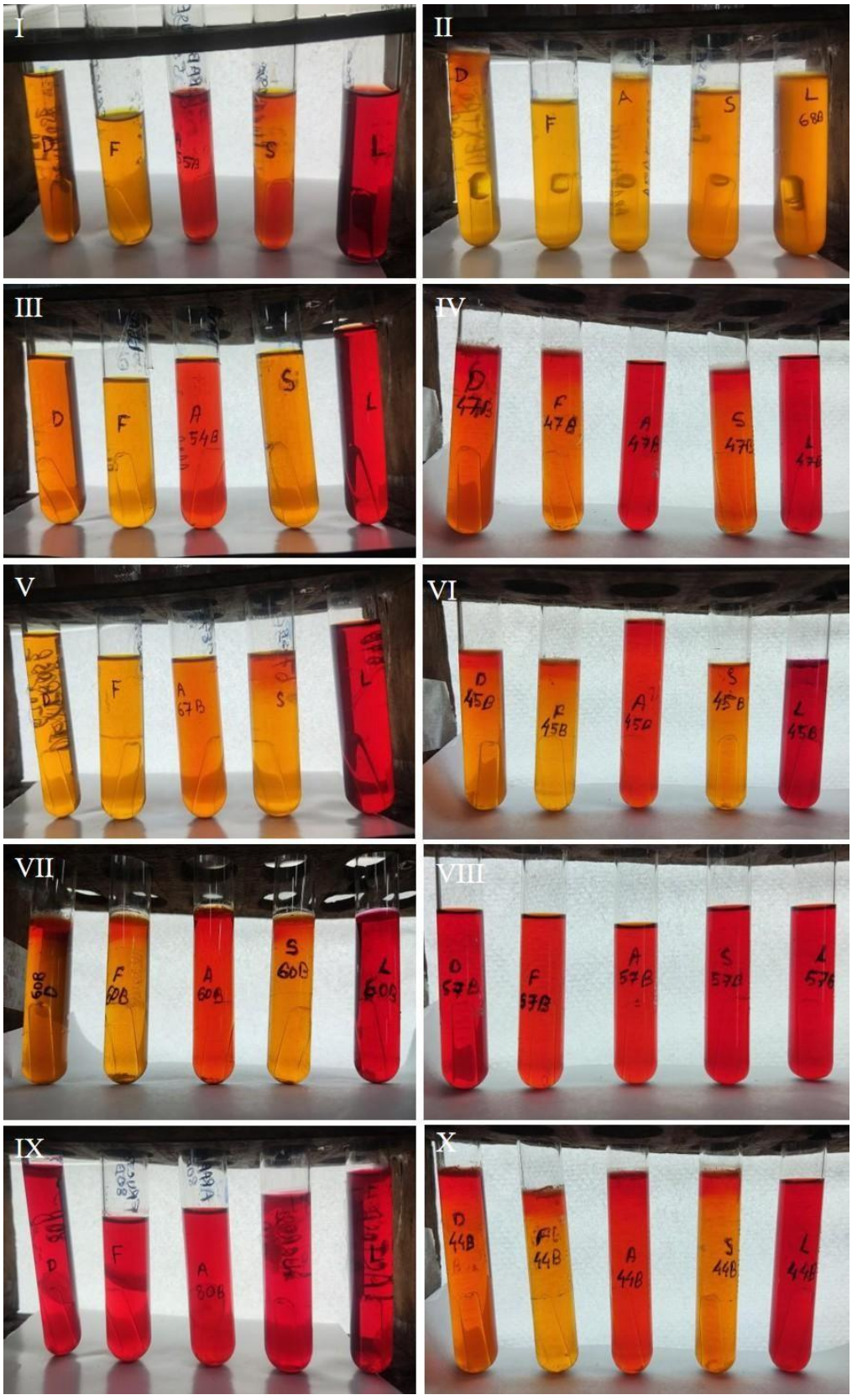

Fig 15: i-x Represents the carbohydrate Utilization test of all ten sample isolates. i- B55, ii- B68, iii- B54, iv- B47, v- B67, vi- B45, vii- B60, viii- B57, ix- B80, x- B44

Table 4 : Biochemical, enzymatic and carbon source utilization by sample isolates

Bacterial No.				B44	B45	B47	B54	B55	B57	B60	B67	B68	B80
Biochemical Test	1	Indole Test		—	—	—	—	—	—	—	—	—	—
	2	Methyl Red Test		—	—	—	—	—	—	—	+	—	+
	3	Vogues-proskaur test		+	+	+	+	+	+	+	+	+	+
	4	Citrate Utilization Test		+	+	+	+	+	+	+	+	+	+
	5	Amylase Test		+	+	+	—	—	—	+	—	+	—
	6	Cellulose utilization Test		—	+	+	+	+	—	+	—	—	—
	7	Carbon Source utilization	Dextrose	+	+	+	+	+	—	+	+	+	—
			Fructose	+	+	+	+	+	—	+	+	+	—
			Arabinose	—	—	—	—	—	—	—	+	+	—
			Sucrose	+	+	+	+	+	—	+	+	+	—
			Lactose	—	—	—	—	—	—	—	—	+	—
	8	Urease Test		+	+	+	+	+	+	+	+	+	+

◆ **Urease test :** All the sample isolates were tested for urease production according to the method outlined by (Brink, 2010), mentioned earlier in the Materials and methods portion. All the ten sample isolates showed positive result in the test (Fig - 16). The results were recorded as positive (+) and negative (-) (Table 4).

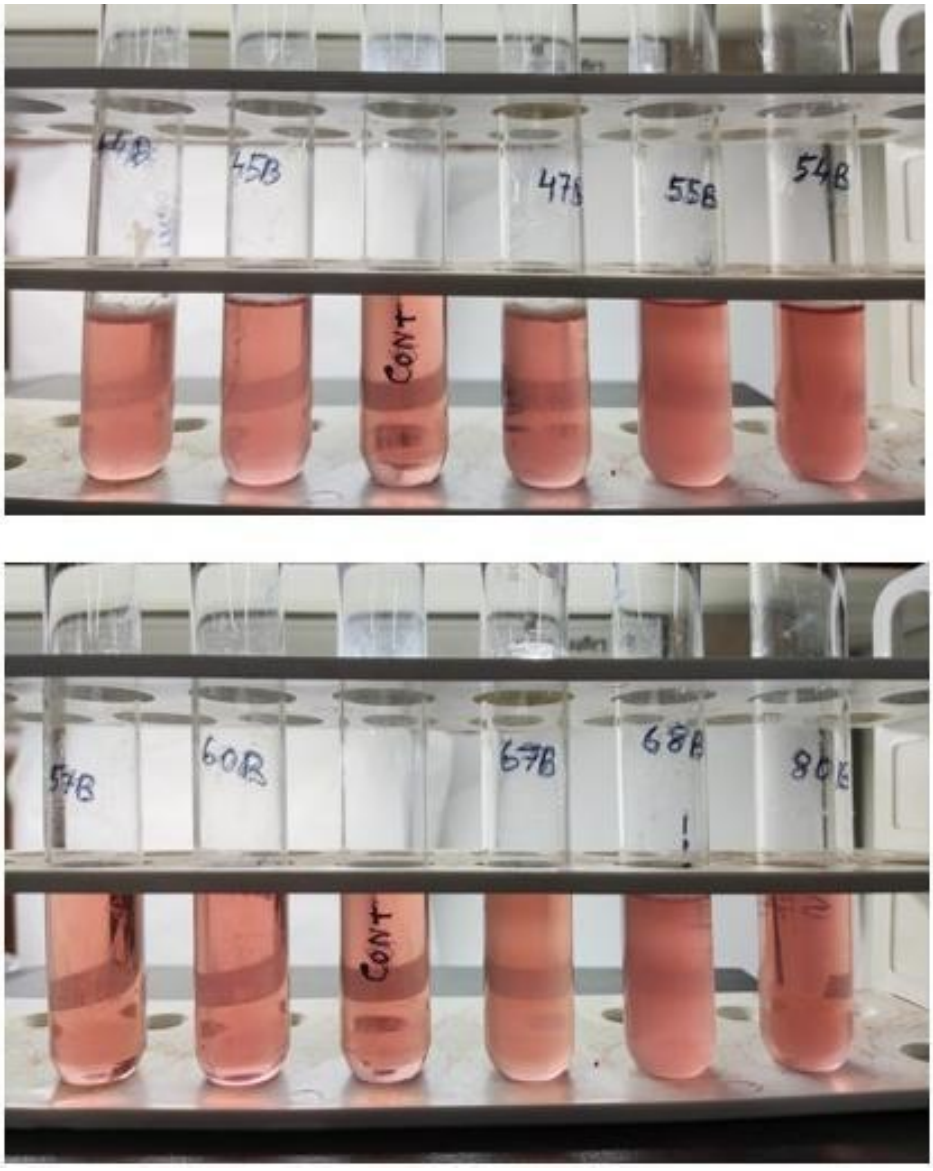

Fig 16 : Urease test results

◆ **Triple sugar iron agar test :** Triple Sugar Iron Agar fermentation by bacterial sample isolates were performed by the method outlined by (Lehman, 2005), mentioned earlier in the Materials and Methods section.B57, B80, B67 showed yellow slant and yellow butt indicating fermentation of all sugars. Only glucose fermentation was indicated by the red slant and yellow butt in B54, B55, B47, B60, B45, B68, and B44 (Fig - 17).

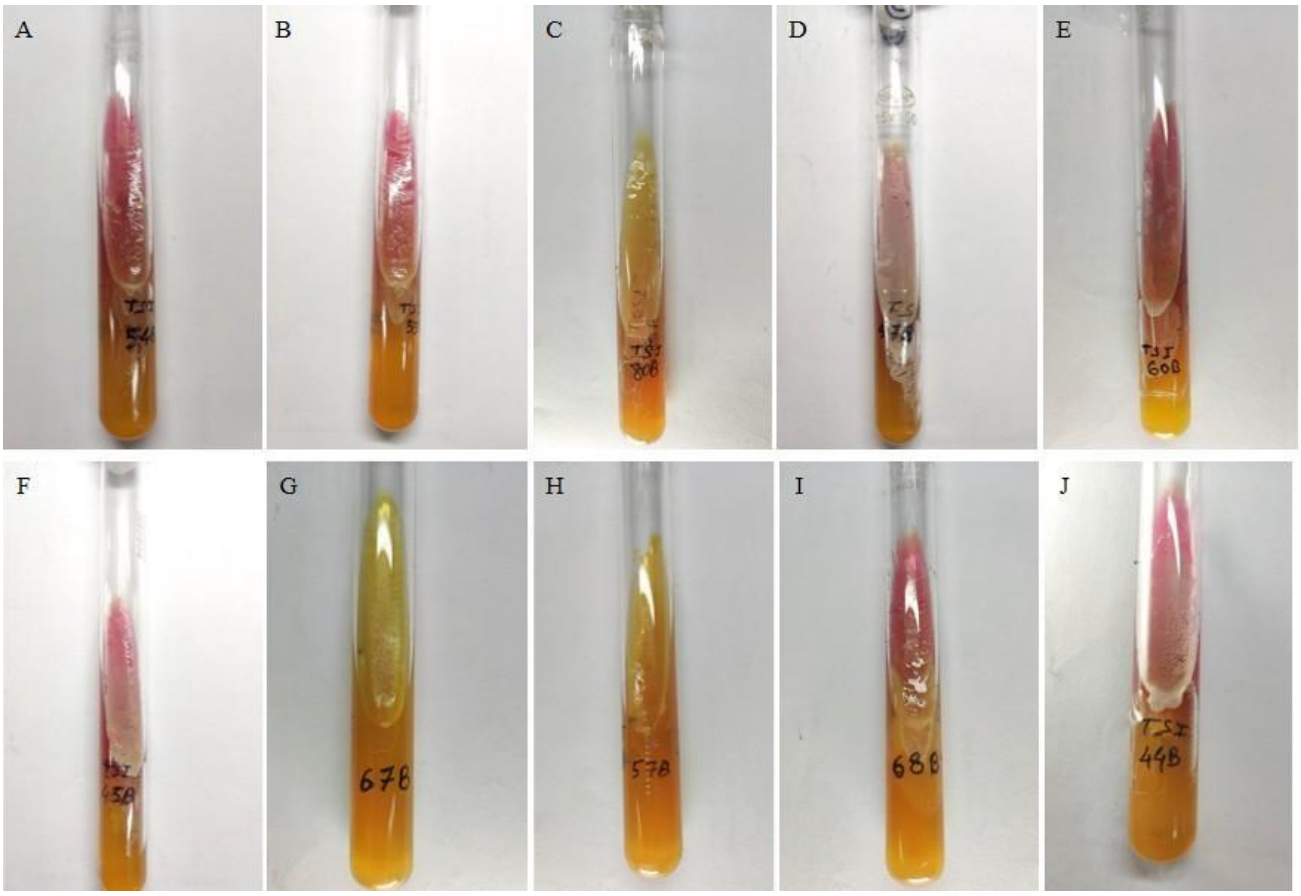

Fig 17: Triple Sugar Iron Agar test results of ten sample isolates

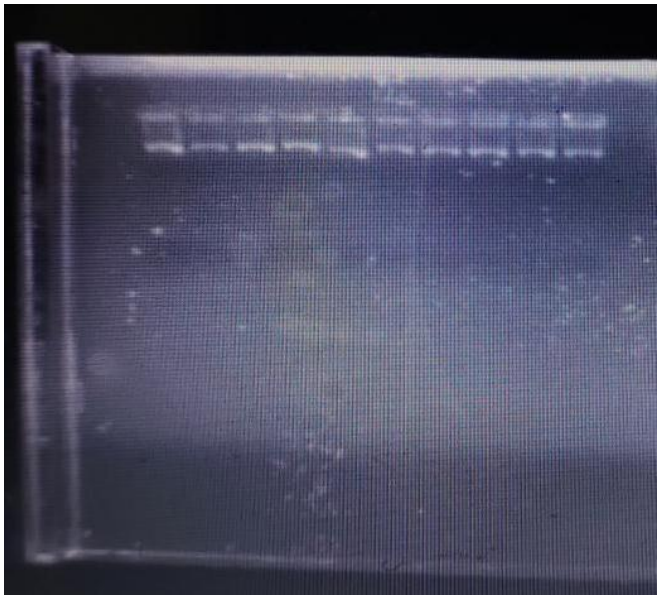

Fig 18 : 16S rRNA bands of isolated samples after PCR amplification

DISCUSSION

Organic farming, including the use of biofertilizers, have been shown to be a promising alternative of chemical fertilizers due to their widespread harmful effects on human being and ecosystem. This approach addresses the escalating demand for nutritious food sources, promotes long-term ecological sustainability, and mitigates issues related to environmental contamination (Mondelaers, 2009; Reganold, 1998; Costa, 2014). Many research work has done with soil inhabiting bacteria that can be used as bioinoculants for the production of biofertilizers. This living bioformulation has a huge potential to replace chemical fertilizers and does not cause any harmful effect on plants and soil.

In this study ten bacterial isolates were studied which were randomly isolated previously from rhizospheric soil of Purulia and Burdwan district of West Bengal. The bacteria were screened for their ability to solubilize zinc, potassium, phosphate and nitrogen fixing abilities. The isolates were then characterized according to Bergey's Manual of Determinative Bacteriology. All of the bacteria showed nitrogen fixing abilities, few of them showed phosphorus, potassium and zing solubilizing abilities. The result of the present work is supported by many similar kinds of works. Sharma et al., (2013 and 2016) showed the action of phosphate and potassium solubilizing microbes in soil to ameliorate the deficiency of both macronutrients in soil. Dent et al. (2018) and Delwich ei al., (1956) showed, respectively the role of non-symbiotic and non-nodular symbiotic soil bacteria to fix nitrogen in soil. The nitrogen fixation ability of all bacterial isolates in the present work was cleared from their growth in nitrogen free media. Ten bacterial species were able to produce ammonia and indole acetic acid which proved their plant growth promoting trait. Similar kind of work was performed by Ahmad et al., (2008) who screened free living rhizospheric bacteria for their plant growth promoting activity. The line of work to characterize the bacterial genera is very similar to the work performed by Holt et al., (1994).

CONCLUSION

Soil is a natural reservoir of a diverse group of bacterial species. Therefore, isolation and characterization of soil bacteria can be mentioned as an important area of research as many of such bacterial isolates can be used as bio inoculants for production of biofertilizers. In this study ten bacterial isolates were checked for their nitrogen fixing, zinc solubilizing, potassium solubilizing and phosphate solubilizing activities. All showed nitrogen fixing abilities, few of them could solubilize phosphorus, potassium and zing. All bacteria produced ammonia and indole. They also produced capsule and some also formed endospore. The results of the above research work says that all the bacterial isolates can be uses as bioinoculant for commercial formulation of biofertilizer.

The results obtained from bacterial characterization indicates that most of the isolates might be of genus *Bacillus.* For further analysis the Genomic DNA of the isolates were extracted. The PCR products were sent for sequencing and for further molecular identification of the isolates.

APPENDIX

- **Nutrient Agar Composition**

Ingredient	g / L
Peptone	5.000
Sodium chloride	5.000
HM peptone	1.500
Yeast extract	1.500
Agar	20.000

- **Nutrient Broth Composition**

Ingredient	g / L
Peptone	5.000
Sodium chloride	5.000
HM peptone	1.500
Yeast extract	1.500

- **Zinc Solubilizing Test Media Composition**

Ingredient	g/ L
Dextrose (Glucose)	10.000
Ammonium sulphate	1.000
Potassium chloride	0.200
Dipotassium hydrogen phosphate	0.100
Magnesium sulphate, heptahydrate	0.200
Zinc oxide	1.000

- **Aleksandrov Agar Composition**

Ingredient	g / L
Magnesium sulphate	0.500
Calcium carbonate	0.100
Potassium alumino silicate	2.000
Dextrose (Glucose)	5.000
Ferric chloride	0.005
Calcium phosphate	2.000
Agar	20.000

- **Pikovskaya Agar Composition**

Ingredient	g / L
Yeast extract	0.500
Dextrose	10.000
Calcium phosphate	5.000
Ammonium sulphate	0.500
Potassium chloride	0.200
Magnesium sulphate	0.100
Manganese sulphate	0.0001
Ferrous sulphate	0.0001
Agar	20.000

- **Jensen's agar Media Composition**

Ingredient	g / L
Sucrose	20.000
Dipotassium hydrogen phosphate	1.000
Magnesium sulphate	0.500
Sodium chloride	0.500
Ferrous sulphate	0.100
Sodium molybdate	0.005
Calcium carbonate	2.000
Agar	20.000

- **Luria - Bertani Broth composition**

Ingredient	g / L
Tryptone	10.000
Yeast extract	5.000
Sodium chloride	10.000
Final pH (at 25 °C)	7.5 ± 0.2

- **Salkowaski reagent Preparation**: Dissolve 12 g of ferric chloride ($FeCl_3$) in 1 liter of concentrated H_2SO_4.

- **Glucose-Peptone Broth Composition**

Ingredient	g / L
Yeast extract	10.0
Bacto peptone (Difco)	20.0
Glucose	10.0
Calcium carbonate ($CaCO_3$)	10.0

- **Citrate Broth Composition**

Ingredient	g / L
Sodium ammonium phosphate	1.500
Magnesium sulphate	0.200
Monopotassium phosphate	1.000
Sodium citrate	3.000

- **MSM Broth Composition**

Ingredient	g / L
Sodium chloride	0.500
Potassium dihydrogen phosphate	3.000
Magnesium sulphate	0.120
Calcium chloride dihydrate	0.013
Yeast extract	3.000
Disodium hydrogen phosphate	6.000

- **Triple Sugar Iron Agar Composition**

Ingredient	g / L
Peptone	20.000
HM extract	3.000
Yeast extract	3.000
Lactose	10.000
Sucrose	10.000
Glucose (Dextrose)	1.000
Iron(III) citrate	0.300
Sodium chloride	5.000
Sodium thiosulphate	0.300
Phenol red	0.024
Agar	12.000

- **Urea Broth Composition**

Ingredient	g / L
Potassium dihydrogen phosphate	9.100
Di-potassium hydrogen phosphate	9.500
Yeast extract	0.100
Phenol red	0.010

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